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14. ABSTRACT

The purpose of the CoE is to discover the mechanism(s) of estrogen-induced apoptosis in advanced antihormone-resistant breast cancers using our unique models, and to establish the clinical value of short-term low-dose estrogen to reverse antihormone resistance in patients exhaustively treated with antihormone therapy. The clinical trial has begun enrolling patients, but enrollment has been low. We have addressed this by amending patient eligibility and expanding trial site locations. We have completed gene expression microarray hybridizations covering extended E2-treatment time courses of wild-type MCF-7:WS8, and of estrogen deprivation-resistant MCF-7:5C and MCF-7:2A cells, which undergo E2-induced apoptosis. Multiple custom methods have been developed for analyses of time-course microarray data. We have also conducted proteomic analyses, and identified proteins which differentially co-immunoprecipitate with the co-activator AIB1 or phospho-tyrosine complexes in an E2-dependent manner. Using the gene expression microarray and proteomic data, networks were built that highlight differential growth versus apoptosis pathways regulated by E2. Further, based on the microarray analyses, we investigated the G protein coupled-receptor GPR30, and the endoplasmic reticulum stress-associated factors caspase-4 and XBP1 in E2-induced growth or apoptosis. Finally, we surprisingly found that long-term treatment of MCF-7:5C cells with the c-Src inhibitor PP2 reversed E2-induced apoptosis.

15. SUBJECT TERMS

Gene expression microarrays, proteomics, GPR30 (also termed GPER), caspase-4, c-Src inhibitor PP2, XBP1

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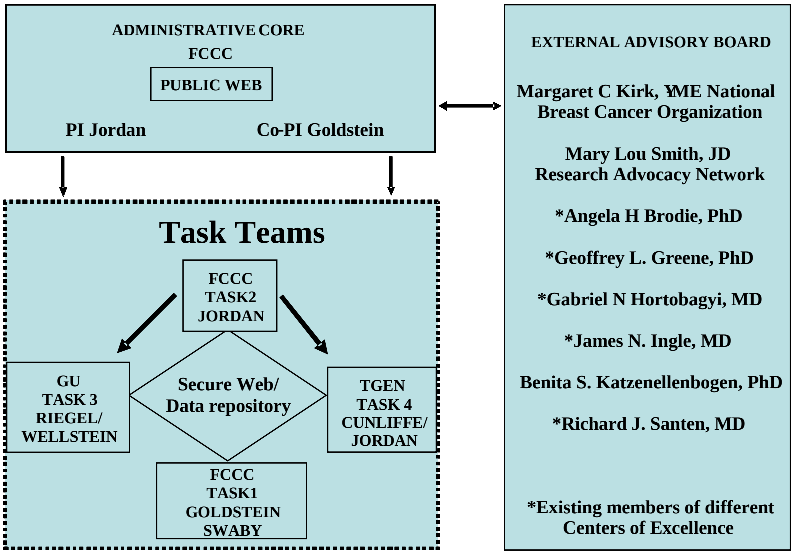
INTRODUCTION

The Center of Excellence Grant is completing four independent, interconnected and synergistic tasks to achieve the goal and answer the overarching question: **to discover the mechanism of estrogen induced breast cancer cell apoptosis and establish the clinical value of short-term low dose estrogen treatment to cause apoptosis in antihormone resistant breast cancer.** To achieve the goal, we had established an integrated organization (Fig. 1) with a first class advisory board that links clinical trials (**Task 1)** with laboratory models and mechanisms (**Task 2)** proteomics **(Task 3)** and genomics **(Task 4)**.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Task Teams
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Figure 1. Organization of the COE up to June 30th, 2009.

Changes in the Organization of the COE

On June 30th, 2009, Dr. Jordan resigned from his position at the Fox Chase Cancer Center. On July 1st, 2009, Dr. Jordan assumed the responsibility of Scientific Director and Vice Chairman of the Department of Oncology at the Lombardi Comprehensive Cancer Center, Georgetown University, Washington, D.C. The reason for the move at this time was the critical mass of outstanding breast cancer medical scientists who were in position at the Lombardi Comprehensive Cancer Center. The move to the Lombardi Cancer Center is optimal, as Drs. Wellstein and Riegel are COE Investigators conducting the proteomics research using our biological samples. The Fox Chase Cancer Center has already written the letter of relinquishment, and the Lombardi Cancer Center is in the process of submitting the requirements for the transfer of the grant there. This move will streamline our communications. The clinical trial, partly funded by the Department of Defense but primarily sponsored by a grant to Dr. Ramona Swaby from AstraZeneca, will continue at Fox Chase Cancer Center. The sub-contract in the Center of Excellence Grant to the Translational Genomics Research Institute (TGen) will continue from the Lombardi Cancer Center. Hosting of the COE website and the secured ftp site for data transfer will transition to GU (Fig. 2).

Dr. Jordan's move to GU has resulted in appointment changes of his staff. Dr. Joan Lewis-Wambi has been appointed as a Research Assistant Professor and remains at Fox Chase Cancer Center. Dr. Eric A. Ariazi has been appointed as Scientific Consultant to Dr. V. Craig Jordan and the Center of Excellence BC050277. Drs. Ping Fan and Surojeet Sengupta have been appointed as Research Assistant Professors at GU and are continuing staff members in Dr. Jordan's laboratory. Ms. Helen Kim has been promoted to Dr. Jordan's Laboratory Manager at GU.

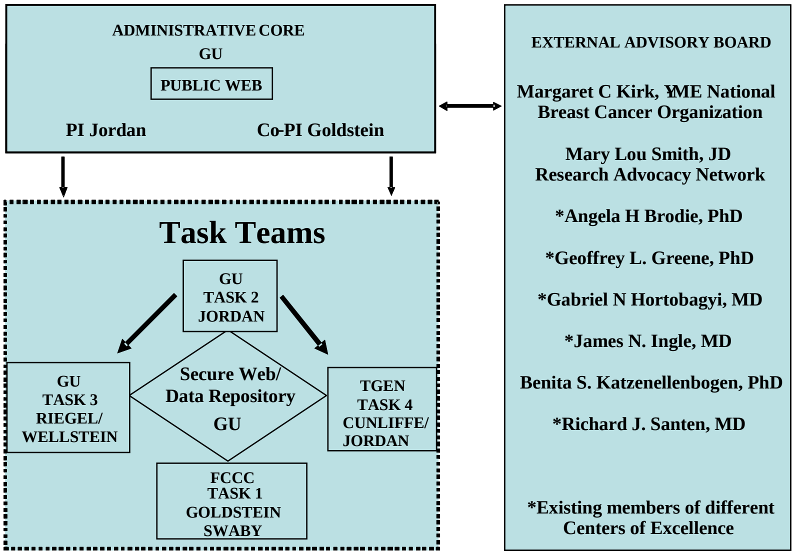
ADMINISTRATIVE CORE
GU
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Figure 2. Organization of the COE after July 1, 2009. Dr. Jordan and his lab have moved to Georgetowen University (GU). Therefore the main site of the CoE has transitioned to GU, including the administrative core and Task 2 (models and mechanisms). The clinical trial in Task 1 remains at Fox Chase Cancer Center (FCCC), and the continuing genomics work in Task 4 remains at TGen (with support of Task 4 by the Jordan lab at GU). GU will host the COE website and the secured data repository.

BODY

Task 1: (FCCC/Swaby, Goldstein) - To conduct exploratory clinical trials to determine the efficacy and dose response of pro-apoptotic effects of estrogen [Estrace] in patients following the failure of two successful antihormonal therapies.

Task 1a: (Swaby and Goldstein) - To confirm the efficacy of standard high dose estrogen (Estrace) therapy and then determine a minimal dose to induce tumor regression.

Here we report work completed on Tasks 1a at the Fox Chase Cancer Center (FCCC) site during year 3 of this COE.

Clinical trial conducted by Ramona Swaby MD, under direction of Lori Goldstein MD at FCCC.

DOSE DE-ESCALATION OF ESTROGEN (ESTRACE) TO REVERSE ANTIHORMONE RESISTANCE IN PATIENTS ALREADY EXHAUSTIVELY TREATED WITH ANTIHORMONE THERAPY

WORK ACCOMPLISHED

During the third year of funding, we have been actively recruiting patients. Seven additional patients have been screened, including one additional patient enrolled. Two patients have been enrolled and treated since the study was activated on 4-25-08. There have been no dose limiting toxicities (DLTs) and/or Serious Adverse Events (SAEs). No patient tissue samples have been obtained as proposed in Task 1b to date due to: 1) patient safety – the patient was on chronic therapeutic anti-coagulant therapy, and 2) an inability to obtain tissue despite biopsy attempt.

In an effort to enhance enrollment, the eligibility criteria was amended previously (August 2008). Barriers to eligibility have included requiring sequential anti-estrogen treatments and excluding patients with bone only disease (*i.e.* – evaluable disease). We have amended the protocol to still retain the estrogen responsiveness (24-months in an adjuvant setting before progression, or at least 6-months in the metastatic setting). We no longer require the two most recent treatments prior to enrollment to be endocrine agents. We are currently in the process of again broadening eligibility to address enrollment by 1) considering patients with evaluable, rather than measurable disease, and 2) allowing pre-menopausal women the opportunity to participate as long as they are rendered post-menopausal by medical ovarian suppression. Lastly, we are amending the exclusion criteria to allow patients who have had chemotherapy or radiotherapy $\leq$ 2 weeks prior to entering the study, and those who have not recovered from any (serious) adverse events to $\geq$ grade 1 toxicity due to agents administered > 2 weeks earlier.

To proactively avoid feasibility issues as well as any obvious safety issues, we deliberately waited to extend the clinical trial to other sites until we had treated at least one patient on trial at Fox Chase Cancer Center, the main clinical site. Originally, it was planned that the study would be extended to Johns Hopkins University as well as to selected FCCC Partners at participating sites. Johns Hopkins University is no longer a planned study site. However, Georgetown University and its clinical trial network, MedStar, have replaced Johns Hopkins University as an academic partner. Regulatory negotiations and approval to open the clinical trial at Georgetown University are currently underway. Dr. Claudine Isaacs is leading the clinical effort at Georgetown University and anticipates enrolling 5 patients annually. Additionally, two Fox Chase Partner network sites have been identified and are also in the process of opening the clinical trial and anticipate enrolling 5 patients annually per site. It is anticipated that these sites will be open to enrollment by the first quarter of 2010.

It is anticipated that by both broadening the eligibility criteria as well as increasing the number of participating sites, we will increase accrual and be able to successfully complete the clinical trial in a timely fashion.

TASK 2: (FCCC/Jordan) - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2a: (Ariazi and Jordan) - To complete a series of experiments using sets of well defined breast cancer models of E_2-induced survival and apoptosis *in vivo* and *in vitro* [at the Fox Chase Cancer Center (FCCC)]. FCCC will generate protein samples for proteomic analyses [carried out] under Task 3 [at Georgetown University (GU)] and RNA samples for gene expression microarray analyses [carried out] under Task 4 [at Translational Genomics Research Institute (TGen)].

Here we report work completed on Task 2a at the Fox Chase Cancer Center (FCCC) site during year 3 of this COE.

Studies carried out at FCCC by Eric Ariazi PhD, in the laboratory of Dr. Jordan.

GENERATION OF CELL LINE SPECIMENS FOR PROTEOMICS AND GENE EXPRESSION MICROARRAY ANALYSES

WORK ACCOMPLISHED

Experiments Completed During the Prior Year 1 (described in the Year 1 Progress Report)

Proteomic Samples for Task 3 and shipped to GU
Experiment 1) Production of MCF-7/WS8 protein samples for proteomics of cells treated plus/minus 10^{-9} M E_2 for a long-term time course in which cells were harvested at 24 h, 48 h, and 72 h.

Experiment 2) Production of MCF-7/5C protein samples for proteomics of cells treated plus/minus 10^{-9} M E_2 for a long-term time course in which cells were harvested at 24 h, 48 h, and 72 h.

Microarray Samples for Task 4 and shipped to TGen
Experiment 3) Production of MCF-7/WS8 RNA samples for microarrays of cells treated plus/minus 10^{-9} M E_2 for 2 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h.

Experiment 4) Production of MCF-7/5C RNA samples for microarrays treated plus/minus 10^{-9} M E_2 for 2 h, 6 h, 12 h, 24 h, 48 h and 96 h.

Experiments Completed During the Prior Year 2 (described in the Year 2 Progress Report)

Proteomic Samples for Task 3 and shipped to GU
Experiment 1) Production of MCF-7/WS8 protein samples for proteomics of cells treated plus/minus 10^{-9} M E_2 for 2 h.

Experiment 2) Production of MCF-7/5C protein samples for proteomics of cells treated plus/minus 10^{-9} M E_2 for 2 h.

Experiment 3) Production of MCF-7/2A protein samples for proteomics of cells treated plus/minus 10^{-9} M E_2 for 2 h.

Microarray Samples for Task 4 and shipped to TGen
Experiment 4) (Short-term time course) Production of MCF-7/2A RNA samples for microarrays of cells treated plus/minus 10^{-9} M E_2 for a short-term time course in which cells were harvested at 2 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 96 h.

Experiment 5) (Long-term time course) Production of MCF-7/2A RNA samples for microarrays treated plus/minus 10^{-9} M E_2 for a long-term time course in which cells were harvested at 3 days, 4 days, 5 days, 6 day, 7 days, 8 days, and 9 days.

Experiments Completed During the Current Year 3 (described below)

Proteomic Samples for Task 3 and shipped to GU

Set 1) To use for identification of ERα-interacting proteins by immunoprecipitation.

Cells were treated ± 10^{-9} M E_2 for 30 minutes.
Experiment 10/20/08) MCF-7/5C cells, 32 × 15cm plates total, 16 plates per treatment
Experiment 10/24/08) MCF-7/WS8 cells, 32 × 15cm plates total, 16 plates per treatment
Experiment 10/28/08) MCF-7/2A cells, 32 × 15cm plates total, 16 plates per treatment

Set 2) To use for identification of tyrosine-phosphorylated proteins by immunoprecipitation.

Cells were treated plus/minus 10^{-9} M E_2 for 30 minutes.

Experiment 12/5/08) MCF-7/WS8 cells, 32 × 15cm plates total, 16 plates per treatment
Experiment 12/12/08) MCF-7/2A cells, 32 × 15cm plates total, 16 plates per treatment
Experiment 12/16/08) MCF-7/5C cells, 32 × 15cm plates total, 16 plates per treatment

Set 3) To use for identification of ERα phosphorylation sites by mass spectrometry analysis of immunoprecipitated ERα.

Cells were treated with 10^{-9} M E_2 for 30 minutes.
Experiment 5/12/09) MCF-7/5C cells, 40 × 15cm plates total
Experiment 5/15/09) MCF-7/WS8 cells, 40 × 15cm plates total
Experiment 5/19/09) MCF-7/2A cells, 40 × 15cm plates total

All samples produced for Year 3 were protein lysates for proteomic analyses, as we had completed generating all RNA samples from cell lines to use for gene expression microarray analyses in prior years.

Cell Lines and Culture Conditions

The cell lines used to generate microarray and proteomics samples were wild-type estrogen-responsive MCF-7/WS8 cells (1, 2), estrogen deprivation-resistant MCF-7/5C cells (1, 3) which undergo E_2-induced apoptosis with fast kinetics (starts within 3 days), and estrogen deprivation-resistant MCF-7/2A cells, which undergo E_2-induced apoptosis with slow kinetics (starts within 7 days) (2, 4).

MCF-7/WS8 cells were maintained in fully-estrogenized media (phenol red-containing RPMI-1640 and 10% whole fetal bovine serum (FBS), supplemented with 6 ng/ml insulin, 2 mM glutamine, 100 μM non-essential amino acids, and 100 U of penicillin and streptomycin per ml). MCF-7/5C and MCF-7/2A cells were maintained in estrogen-free media (phenol red-free RPMI-1640 and 10% dextran-coated charcoal-treated FBS (DCC-FBS) plus the same supplements as for fully-estrogenized media). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

All experiments were conducted in estrogen-free RPMI-1640 media plus 10 % DCC-FBS. Also, MCF-7/WS8 cells were switched from fully-estrogenized media to estrogen-free media three days prior to an experiment.Cells were seeded into 15-cm plates at 80% confluency. The day following seeding, cells were treated with or without 10^{-9} M E_2 for 30 m as appropriate and incubated 37° C in a humidified 5% CO_2 atmosphere. At the end of the 30 m incubation, cells were collected and protein lysates prepared with all steps carried out at 4°C. Cells were washed in plates with ice-cold phosphate-buffered saline (PBS), collected by scraping, transferred to a microcentrifuge tube and pelleted at 5,000 rcf for 15 s. The PBS supernatant was aspirated, and protein lysates prepared by resuspending the cells in 150 µl of RIPA lysis buffer (Sigma-Aldrich, Cat. No. R0278) supplemented with protease inhibitors (Roche; Cat. No. 11836153001) and phosphatase inhibitors [Phosphatase Inhibitor Cocktail Sets I and II; Calbiochem; Cat. Nos. 524624 and 524625, respectively]. Cells which had been E_2-treated were maintained in 10^{-9}M E_2 final concentration during the collection and protein lysate preparation procedure by supplementing the PBS and RIPA buffer 1:1,000 (*v*/*v*) with 10^{-6} M E_2. Proteins were allowed to extract into the lysis buffer by end-over-end rotation for 30 m at 4°C. Cellular debris was pelleted by centrifugation at 14,000 rcf for 10 m at 4°C, and cleared lysates were transferred to coded vials and shipped to Georgetown University for proteomic analysis. At least 2 mg of protein per 15 cm plate was collected.

Validation of posttranslational modifications (phosphorylation) of estrogen receptor α (ERα) for Task 3

The third set of protein lysates generated (experiments 5/12/09, 5/15/09, 5/19/09) were purposed for identification of ERα phosphorylation sites by mass spectrometry analysis of immunoprecipitated

ERα under Task 3. To confirm that E_2 induced phosphorylation of ERα, additional cells from all three cell lines were treated plus/minus 10^{-9} M E_2 for 30 m. Protein lysates were analyzed by immunoblotting for phospho-Ser118 ERα and total ERα. The phospho-Ser118 ERα antibody detected 2 bands (green), but eletrophoretic co-localization with total ERα (red) indicated the specific phospho-Ser118 ERα band. The immunoblot analysis showed that E_2 treatment led to increased levels of phospho-Ser118 ERα.

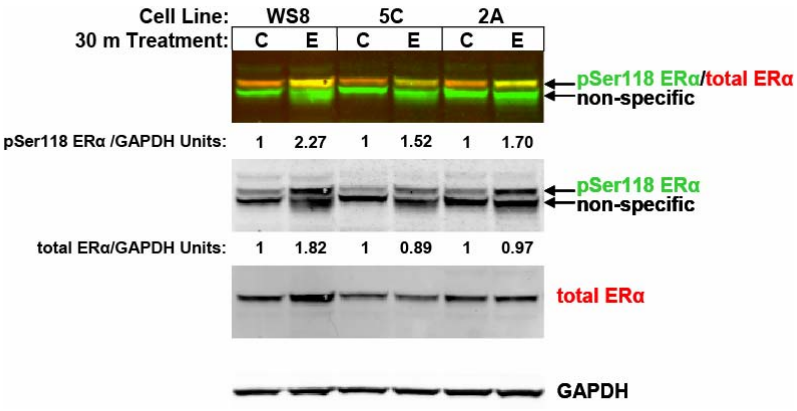
Cell Line: WS8 5C 2A
30 m Treatment: C E C E C E
pSer118 ERα/total ERα
non-specific
pSer118 ERα /GAPDH Units: 1 2.27 1 1.52 1 1.70
pSer118 ERα
non-specific
total ERα/GAPDH Units: 1 1.82 1 0.89 1 0.97
total ERα
GAPDH

Figure 1. E_2-induced phosphorylation of ERα at Ser118 by immunoblotting. MCF-7:WS8, MCF-7:5C and MCF-7:2A cells were control- (C) or 10^{-9} M E_2–treated (E) for 30 m. The immunoblot was probed for phospho-Ser118 ERα (clone NL44, Millipore, Cat. No. 05-793) and total ERα (Clone AER611, Thermo Scientific/Lab Vision, Cat. No. MS-1071-S). Primary antibodies were detected using infrared-fluorescently–labeled secondary antibodies. The membrane was scanned using an Odyssey Infrared Imaging System (Li-Cor Biosciences; Lincoln, NE). Phospho-Ser118 ERα antibody was visualized in the red channel, and total-ERα antibody in the green channel.

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TASK 2. FCCC/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2b-1: (Ariazi and Jordan) – To confirm and validate developing pathways of E_2-induced breast cancer cell survival and apoptosis.

Task 2b is organized into sub-sections 1-4 according to projects led by senior investigators in Dr. Jordan's laboratory that involve deciphering pathways of E_2-induced breast cancer cell survival and apoptosis.

Here we report work completed on Task 2b-1 at the Fox Chase Cancer Center (FCCC) site during year 3 of this COE.

Studies carried out at FCCC by Eric Ariazi PhD, in the laboratory of Dr. Jordan.

THE G PROTEIN-COUPLED RECEPTOR GPR30 INHIBITS PROLIFERATION OF ESTROGEN RECEPTOR-POSITIVE BREAST CANCER CELLS

Introduction

By examining the Affymetrix platform microarray data that we reported in (1), we observed that the G protein-coupled receptor GPR30 was expressed at higher levels in MCF-7:5C and MCF-7:2A cells compared toMCF-7:WS8 cells. GPR30 is a seven-transmembrane-domain protein that has been identified as a novel (E_2)-binding protein structurally distinct from the classical estrogen receptors α and β (ERα and ERβ). GPR30 can mediate rapid E_2-induced non-genomic signaling events including mobilization of intracellular calcium (Ca^{2+}) stores, and stimulation of adenylyl cyclase, mitogen-activated protein kinase (MAPK) and phosphatidyl inositol 3-kinase (PI3K) signaling pathways via transactivation of epidermal growth factor receptors [reviewed in (2-6)]. GPR30 also exhibits prognostic utility in endometrial (7) ovarian cancer (8), and breast cancer (9), and can modulate growth of hormonally responsive cancer cells. Therefore, GPR30 likely plays important roles in modulating estrogen responsiveness, and in the development and/or progression of hormonally responsive cancers. Hence, we investigated the potential for GPR30 to modulate E_2-stimulated growth of wild-type MCF-7:WS8 (or simply MCF-7) cells and the estrogen deprivation-resistant MCF-7:5C (or 5C) and MCF-7:2A (or 2A) cells.

WORK ACCOMPLISHED

Initially, to confirm the microarray data, GPR30 mRNA expression was measured by quantitative real-time reverse-transcriptase PCR (qRT-PCR). GPR30 mRNA levels were increased 3.1 and 5.5-fold in 5C and 2A cells, respectively (Fig. 1A) compared to MCF-7 cells. ERα mRNA expression in these cells was also measured and found that it was similarly increased 2.8 and 6.1-fold in the 5C and 2A cells, respectively (Fig. 1A). Hence, the degree of GPR30 overexpression followed the same pattern as that of ERα overexpression. This indicates that not only was ERα selected, but also GPR30 under the selective pressure of estrogen deprivation.

Next whether increased GPR30 expression correlated with increased GPR30 functional activity was evaluated by measuring Ca^{2+} mobilization in response to G-1, a GPR30 agonist that does not bind ERα or ERβ. Ca^{2+} responses were detected using microscopic imaging of cells loaded with the fluorescent Ca^{2+} indicator Fura-2 AM (Fig. 1B). In MCF-7 cells, administration of the GPR30 specific agonist G-1 at 10^{-7} M induced a slow and sustained rise in intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, with a maximal increase of 142 + 1.4 nM (n = 47 cells, *P* < 0.001) at 6 m after administration (Fig. 1B). In 5C cells, 10^{-7} M G-1 induced a maximal 228 ± 2.7 nM (n = 58 cells) increase in $[Ca^{2+}]_i$ by 6 m after administration (Fig 1B); hence this effect was larger in 5C cells than in MCF-7 cells (*P* = 0.0053). Likewise in 2A cells, administration of 10^{-7} M G-1 maximally elevated $[Ca^{2+}]_i$ by 405 ± 3.1 nM (n = 56) after 9 m (Fig. 1B); and this effect was even larger in 2A cells than in 5C cells (*P* = 0.0038). Therefore, 5C and 2A cells exhibit increased GPR30 activity compared to wild-type MCF-7 cells, and this increased activity correlated with GPR30 overexpression.

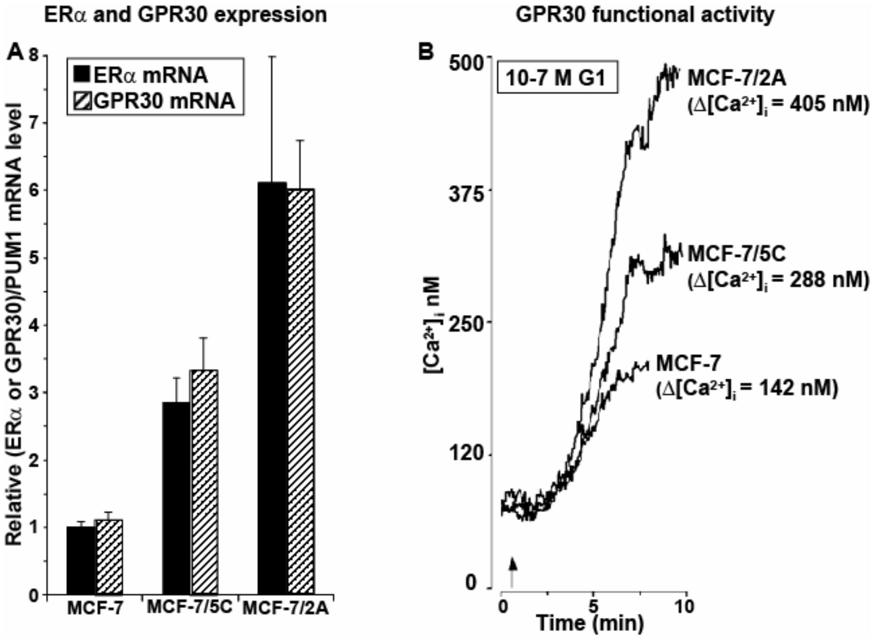

Figure 1. GPR30 is overexpressed and exhibits increased Ca^{2+} mobilization activity in estrogen deprivation-resistant MCF-7/5C and MCF-7/2A cells compared to wild-type MCF-7 cells. (A) ERα and GPR30 mRNA were overexpressed in MCF-7/5C and MCF-7/2A cells vs. MCF-7 cells as measured by qRT-PCR. All cells were assayed under estrogen-free conditions. (**B)** The selective GPR30 agonist G-1 (10^{-7} M) induced significantly greater increases in intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$ in MCF-7/5C (228 ± 2.7 nM, n = 58 cells) and in MCF-7/2A cells (405 ± 3.1 nM, n = 56 cells) than in MCF-7 cells (142 ± 1.4 nM, n = 47 cells). The effects were maximal after 9 m of compound administration in MCF-7/2A cells, and 6 m in MCF-7/5C and MCF-7 cells. $[Ca^{2+}]_i$ was measured by confocal microscopic imaging of cells loaded with the fluorescent Ca^{2+} indicator Fura-2 AM.

Given GPR30's likely involvement in modulating estrogen responsiveness, and that it was overexpressed and showed parallel increases in activity in 5C and 2A cells compared to MCF-7 cells, we elected to carefully investigate it's role in modulating E_2-induced survival and apoptosis in these cells. However, before investigating GPR30 in estrogen deprivation-resistant cells, it was important to initially establish GPR30's functions in wild-type estrogen-responsive MCF-7 cells. We therefore investigated the functional relationship between GPR30 and ERα, and GPR30's role in E_2–stimulated growth of ERα–positive MCF-7 breast cancer cells. First, a previously reported breast carcinoma microarray study was mined to examine the distribution of GPR30 expression in ERα–positive vs. –negative carcinomas. Finding that elevated GPR30 expression associated with ERα–positive breast cancer, the contribution of ERα and GPR30 in several E_2–responsive activities was investigated using a combination of ER and GPR30 selective ligands and small interfering RNA (siRNA)–based methodology. The investigated E_2 responsive activities included regulation of GPR30 expression, intracellular calcium mobilization, cellular growth, and cell cycle progression. The results indicated that in ERα–responsive MCF-7 breast cancer cells, ERα down-regulates GPR30 expression, GPR30 mediates E_2–induced Ca^{2+} mobilization, and GPR30 opposes E_2–stimulated proliferation by blocking cell cycle progression.

Increased GPR30 mRNA expression associated with ERα–positive status in 1,250 breast carcinomas

Evidence of a relationship between GPR30 and ERα expression was sought by mining publicly available and well-annotated gene expression microarray data sets comprising 1,250 breast carcinomas. The data are presented here as five distinct cohorts.

The first cohort was derived from the breast cancer microarray data reported by van de Vijver *et al*. (10) (available at www.rii.com/publications/2002/nejm.html) and is referred to herein as the NKI cohort (n = 295; samples collected at the Netherlands Cancer Institute, Amsterdam). The expression data in the NKI cohort data was obtained using a 2-color 60-polymer oligonucleotide format to which cRNA from one tumor was competitively hybridized against a pooled reference of equal amounts of cRNA from all tumors. Expression values correspond to the normalized $\log_2$ ratio intensity units of the GPR30 probe. The ER status was determined based on the microarray ERα probe intensity ratio of < -0.65 on a $\log_2$ scale, and corresponds to < 10 % of nuclei staining for ERα by IHC (10). In the NKI data set, Pearson's correlation coefficients were computed between GPR30 and all other genes using the R software package (www.R-project.org).

Cohorts 2 through 5 were assembled from publicly available data sets published as part of the GEO (Gene Expression Omnibus) data repository (11). These cohorts are termed herein as follows and consist of the following GEO data sets: the Uppsala cohort (GSE3494/GSE4922/GSE6532, n = 254; samples collected in Uppsala County, Sweden), the Stockholm cohort (GSE1456, n = 159; samples collected at the Karolinska Hospital in Stockholm, Sweden), the EMC cohort (GSE2034/GSE5327, n = 344; samples collected at the Erasmus Medical Center, Rotterdam, Netherlands), and the TRANSBIG cohort (GSE7390, n = 198; samples collected by the translational research network managed by the Breast International Group). The Uppsala and EMC cohorts contain samples processed at the same institution that span multiple GEO accession numbers. All studies comprising these 4 cohorts utilized Affymetrix microarray technology (Santa Clara, CA; www.affymetrix.com), and specifically the HG-U133A/B GeneChips. Where available, raw data (in the form of CEL files) were downloaded, otherwise MAS5.0 normalized data were downloaded (CEL files were available for all studies except GSE2034 and GSE5327). All data pre-processing and normalization were performed using the R software package, and libraries provided via the Bioconductor project (12). To preserve a consistent normalization strategy across all cohorts, raw data were MAS5.0 normalized on a per-cohort basis using the `justMAS` function in the `simpleAffy` library from Bioconductor (no background correction, target intensity of 600). After normalization, gene expression data were extracted for the GPR30 probe 210640_s_at. ER status was available via the Supplementary Information provided by GEO.

The first collection of breast carcinomas examined consisted of the NKI cohort (n = 295). Data in the NKI cohort was collected using 2-color oligonucleotide microarrays, and GPR30 expression data are presented as the $\log_2$ ratio intensity units of the GPR30 mRNA level in a single carcinoma relative to a pooled RNA reference consisting of all the carcinomas (Fig. 2A). Comparison of GPR30 mRNA levels using the non-parametric Mann-Whitney rank sum test showed that GPR30 mRNA levels were significantly higher in ERα–positive compared to ERα–negative tumors ($P < 0.0001$). The upper range of GPR30 expression was 7.7-fold higher on a linear scale in the ERα-positive compared to ERα–negative carcinomas. Evaluation of GPR30 and ERα mRNA levels as continuous variables showed that expression of these genes correlated (Pearson's correlation coefficient $\rho = 0.30$, adjusted for all other gene comparisons $P < 0.0001$).

The second collection of breast carcinomas examined consisted of four independent cohorts that all utilized 1-color Affymetrix oligonucleotide microarrays; these cohorts are indicated in Fig. 2B and GPR30 expression values are presented as MAS5.0 normalized intensity units. GPR30 mRNA levels were again compared using the Mann-Whitney test, and found to be significantly higher in the ERα-positive breast cancers compared to the ER-negative cancers in all 4 cohorts (Uppsala cohort, P = 0.0401, n = 244; Stockholm cohort, P = 0.0091, n = 159; EMC cohort, P = 0.0050, n = 344; TRANSBIG cohort, P = 0.0024, n = 198).

Therefore, increased GPR30 expression levels showed an association with ERα–positive status in 5 independent cohorts comprising 1,250 breast cancers.

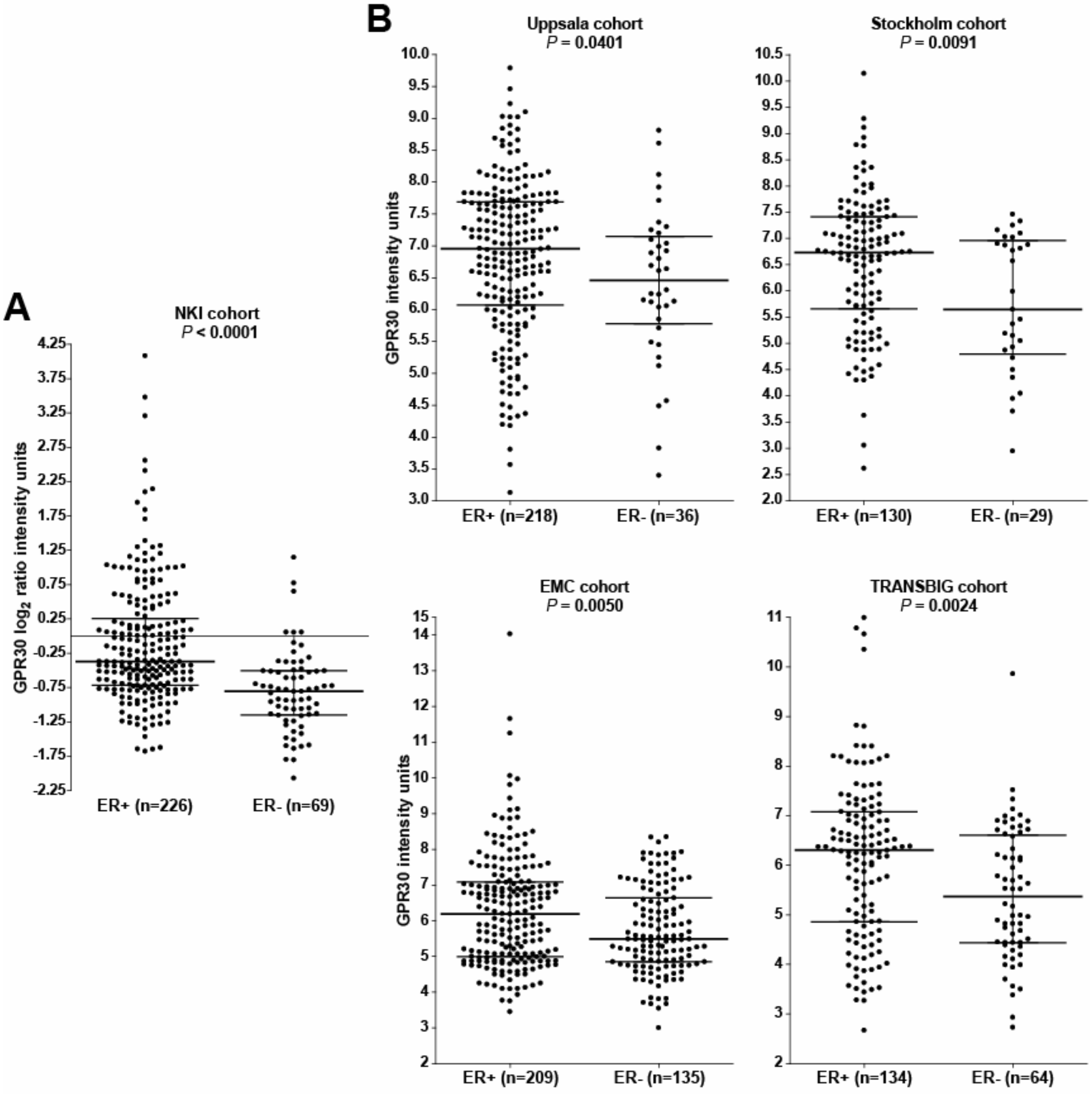

Figure 2. GPR30 mRNA expression shows an association with ERα-positive status in human breast carcinomas. GPR30 expression values and ERα status information were extracted from publicly available gene expression microarray data sets comprising five independent cohorts of 1,250 breast cancers in total. **(A)** GPR30 mRNA levels in the NKI cohort. Expression data in the NKI cohort were collected using 2-color oligonucleotide-format microarrays that were competitively hybridized with cRNA from a single tumor against a reference cRNA pooled from all of the tumors. GPR30 expression levels are shown as normalized $\log_2$ intensity ratio units. **(B)** GPR30 mRNA levels in 4 additional cohorts (Uppsala, Stockholm, EMC, and TRANSBIG). Expression data in these cohorts were collected using 1-color Affymetrix oligonucleotide microarrays. GPR30 expression levels are shown as MAS5.0 normalized intensity units. *A-B,* The numbers of ERα-positive (ER+) and ER-negative (ER–) breast cancers are shown, and the bars from top to bottom indicate the 75th, 50th (median), and 25th percentiles. Comparison of GPR30 mRNA levels versus ERα status was conducted using the non-parametric Mann-Whitney rank test. GPR30 mRNA levels were significantly higher in ERα–positive breast cancers in all 5 cohorts. *P*-values are indicated.

E_2 down-regulated GPR30 mRNA expression via ER and not GPR30

Since ERα and GPR30 shared a statistical relationship in human breast carcinomas, a functional relationship between these genes was further explored using ERα–positive MCF-7 human breast cancer cells. As a first step, GPR30 regulation in response to E_2 was investigated. MCF-7 cells were treated with 10^{-9} M E_2 or control treated (vehicle only) over a 96 h time course, followed by determination of ERα and GPR30 mRNA levels by qRT–PCR. As expected, ERα mRNA levels showed a steady decline reaching a 59% decrease over 96 h in response to E_2 (Fig. 3A). E_2 also led to down-regulation of GPR30, but the kinetics of this down-regulation were faster than that of ERα down-regulation as GPR30 mRNA levels were decreased by 37 % at 2 h ($P = 0.0013$), and by 79 % at 24 h ($P < 0.0001$). Afterwards, GPR30 mRNA levels rebounded such that they were not different from control treatment at 72 and 96 h (Fig. 3B). Therefore, E_2 down–regulated GPR30 mRNA expression in a time–dependent manner. Next, GPR30 mRNA levels in response to 24 h treatment with a serial–dilution series of E_2 was evaluated in MCF-7 cells. GPR30 mRNA expression decreased in a concentration–dependent manner from 10^{-12} M E_2 to 10^{-10} M E_2, and remained repressed from 10^{-9} M E_2 to 10^{-8} M E_2 (Fig. 3C).

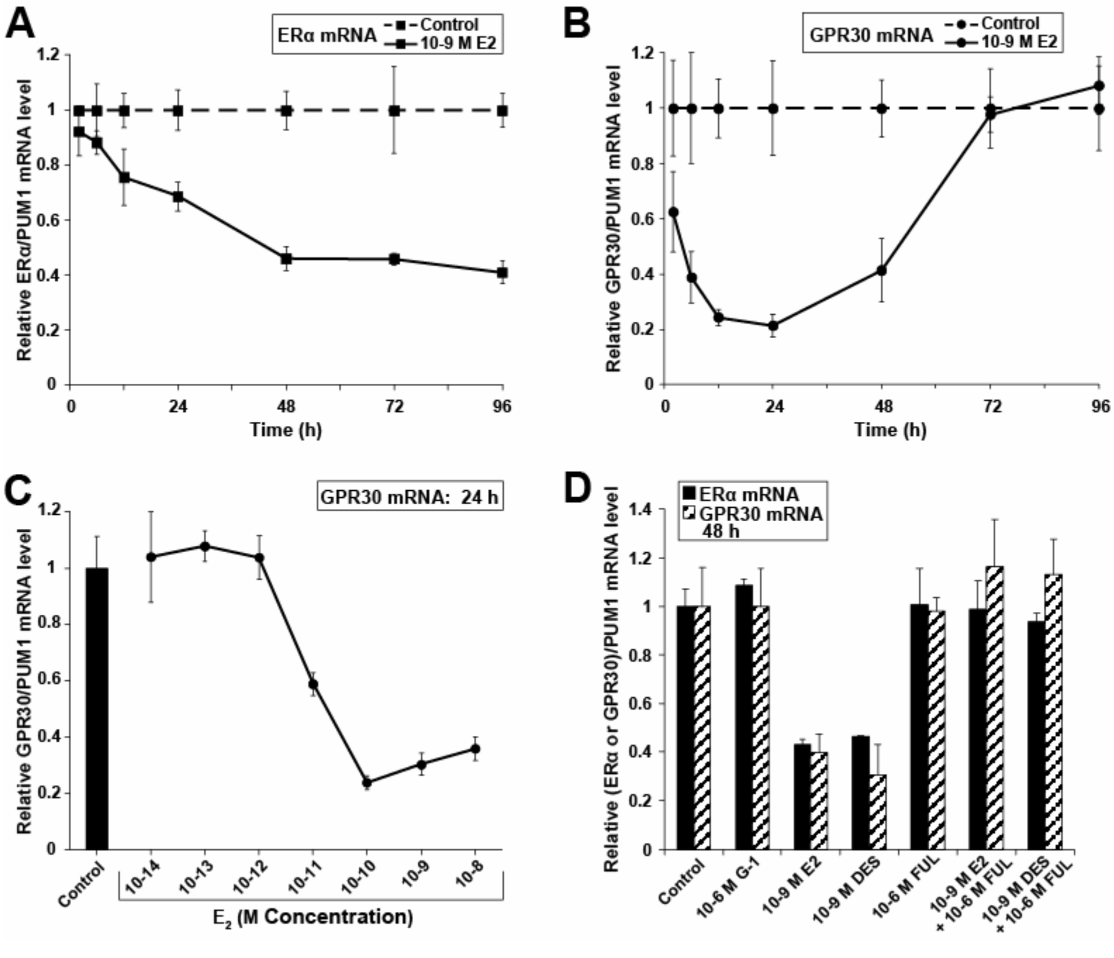

Figure 3. E_2 represses ERα and GPR30 mRNA levels via ER and not GPR30 in MCF-7 cells. E_2-regulation of **(A)** ERα and **(B)** GPR30 mRNA levels across a 96 h time course. MCF-7 cells were treated with 10^{-9} M E_2 or with the vehicle ethanol alone for 2, 6, 12, 24, 48, 72, and 96 h. **(C)** GPR30 mRNA levels in response to a serial dilution series of E_2. MCF-7 cells were treated for 48 h with 10^{-14} M to 10^{-8} M E_2. GPR30 mRNA levels were down-regulated by E_2 in a time- and concentration-dependent manner. **(D)** ERα (solid bars) and GPR30 (hatched bars) mRNA levels in response to 48 h treatment with ER and GPR30 ligands as indicated. DES and not G-1 down-regulated GPR30 and ERα mRNA expression. mRNA levels were determined by qRT-PCR as described in Methods. Each data point represents the average of 6 (*A-B*), or 4 (*C-D*) biological replicates and error bars their SDs.

E_2 is an agonist of both ERα and GPR30; therefore, it was possible that either ERα or GPR30, or both receptors were mediating the effects of E_2 on GPR30 repression. To differentiate these possibilities, MCF-7 cells were treated for 48 h with ligands selective for ER or GPR30 including G-1 which only binds GPR30 as an agonist (13), DES which only binds ERs as an agonist (14), and with FUL which is a pure ER antagonist that also binds GPR30 as an agonist (14) (Fig. 3D). As determined by qRT–PCR, 10^{-6} M G-1 did not alter GPR30 mRNA expression relative to control (vehicle only) treatment. DES at 10^{-9} M repressed GPR30 expression by 54 %, which was very similar to the 57 % repression due to 10^{-9} M E_2. FUL at 10^{-6} M completely blocked the effects of both E_2 and DES. Therefore E_2 likely acted via ER and not GPR30 to down-regulate GPR30 mRNA expression.

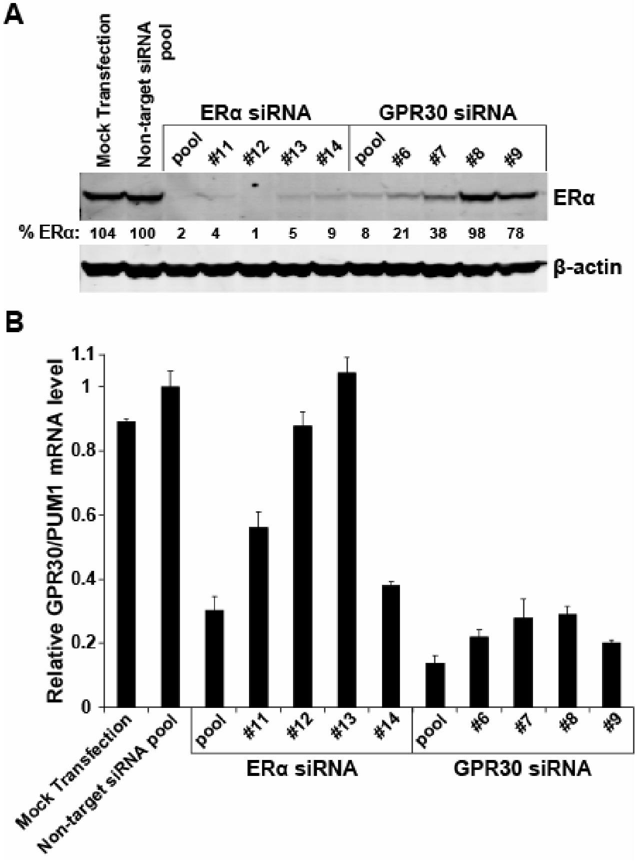

Figure 4. Deconvolution of ERα and GPR30 siRNA pools in ECC-1 endometrial cancer cells. (A) ERα protein levels by immnoblot analysis and **(B)** GPR30 mRNA levels by qPCR in ERα siRNA and GPR30 siRNA-transfected ECC-1 endometrial cancer cells. ECC-1 cells were transfected and assayed under estrogen-free conditions at 48 h following the transfection. The immunoblot was visualized using a Li-Cor Odyssey infrared scanner. Quantitation of ERα protein levels normalized to β-actin are indicated. GPR30 mRNA levels represent the average of 4 biological replicates and error bars their associated SDs. Testing of individual siRNAs indicated that only ERα #13 and GPR30 #8 siRNA exhibit on-target knockdown without off-target effects.

Knockdown of ERα increases GPR30 functional activity

One of E_2's rapid non-genomic effects is mobilization of intracellular Ca^{2+} stores. The GPR30 specific agonist G-1 also induces rapid mobilization of intracellular Ca^{2+}. To investigate whether ERα and/or GPR30 mediates the Ca^{2+} mobilization in response to E_2 in MCF-7 cells, the relative contribution of each receptor was determined by depleting its expression using RNA interference (RNAi). However, before examining the effect of ERα and GPR30 depletion on Ca^{2+} mobilization, an ERα siRNA pool, a GPR30 siRNA pool, and the individual siRNAs in each pool were evaluated (Fig. 4). Since ERα regulated GPR30 expression in MCF-7 cells (Fig. 3B-C), siRNA–mediated depletion of ERα could potentially alter expression of GPR30. Therefore, evaluation of the ERα and GPR30 siRNAs was carried out in a non–breast cancer cell type. ERα–positive ECC-1 endometrial cancer cells were chosen since we have observed that E_2 does not significantly regulate GPR30 mRNA expression in this cell line (data not shown). ECC-1 cells were transfected with the siRNAs, and 48 h later, ERα protein (Fig. 4A) and GPR30 mRNA expression (Fig. 4B) were determined by semi-quantitative immunoblot analysis and real-time qPCR, respectively. The ERα pool and individual siRNAs (#11 to #14) all effectively depleted ERα by greater than 90 %. Similarly, the GPR30 pool and individual siRNAs (#6 to #9) depleted GPR30 mRNA expression from 86 % to 71 %. However, the ERα pool siRNA, and ERα siRNAs #11 and #14 decreased GPR30 mRNA expression by 70 %, 44 %, and 62 % respectively, while ERα siRNA #13 did not. Likewise, the GPR30 pool siRNA, and GPR30 siRNAs #6 and #7 significantly decreased ERα protein expression by 92 %, 79 %, and 62 %, respectively, while GPR30 siRNA #8 did not. Since the ERα siRNAs led to varying decreases in GPR30 expression, and similarly since the GPR30 siRNAs led to varying decreases in ERα expression, it was concluded that these effects were off–target. Therefore, for all further siRNA–based experiments presented, the ERα siRNA #13 and GPR30 siRNA #8 were employed as these siRNAs exhibited the least off–target effects.

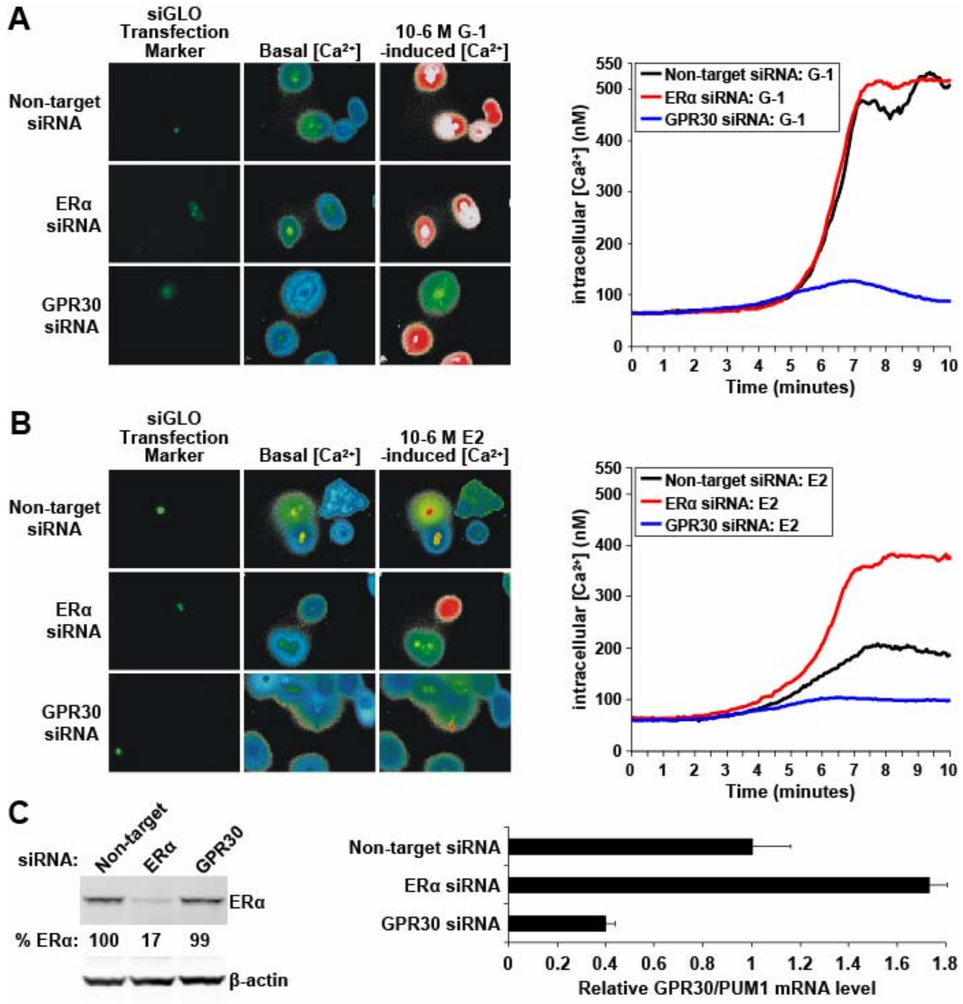

Figure 5. GPR30 and not ERα mediates E_2-induced Ca^{2+} mobilization in MCF-7 cells. Imaging and quantitated traces of **(A)** G-1–induced and **(B)** E_2–induced Ca^{2+} responses. Cells were transfected with non-targeting pool siRNA, ERα (#13) siRNA, and GPR30 (#8) siRNA. Transfected cells were labeled using siGLO Green and present as green cells. Cells were loaded with the fluorescent Ca^{2+} chelator Fura-2AM and intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$ were determined in individual cells using fluorescence microscopy. Low levels of basal $[Ca^{2+}]_i$ are visualized as blue and green fluorescence, while higher levels of $[Ca^{2+}]_i$ are seen as red and white fluorescence. Administration of 10^{-6} M G-1 produced increases in $[Ca^{2+}]_i$ by 463 ± 2.7 nM (n = 19 cells) in non-targeting siRNA-transfected cells, by 471 ± 3.6 nM (n = 14 cells) in ERα siRNA-transfected cells, and by 58 ± 1.3 nM (n = 19 cells) in GPR30 siRNA-transfected cells. Administration of 10^{-6} M E_2 produced increases in $[Ca^{2+}]_i$ by 159 ± 1.6 nM (n = 9 cells) in non-targeting siRNA-transfected cells, by 314 ± 3.2 nM (n = 17 cells) in ERα siRNA-transfected cells, and by 52 ± 0.8 nM (n = 11 cells) in GPR30 siRNA-transfected cells. **(C)** ERα protein levels were measured by immunobloting using a Li-Cor Odyssey infrared scanner, and GPR30 mRNA levels by qPCR in siRNA-transfected cells at 48 h following the transfection. ERα protein and GPR30 mRNA levels were effectively depleted in the appropriate siRNA-transfected cells.

To determine whether E_2–induced Ca^{2+} mobilization was mediated by ERα or GPR30, MCF-7 cells were transfected with ERα (#13) siRNA, GPR30 (#8) siRNA, and a non-targeting siRNA pool as a control. Forty-eight hours following completion of the transfection, changes in intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$ were measured at the single cell level using the fluorescent Ca^{2+} indicator Fura-2AM and microscopic imaging (Fig. 5A-B). Individual transfected cells were marked by co-transfection with the indicator siGLO Green that localizes to the nucleus.

To validate this experimental system, Ca^{2+} mobilization in response to the GPR30 specific agonist G-1 was first evaluated (Fig. 5A). In non-targeting siRNA-transfected cells, 10^{-6} M G-1 induced an increase in intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$ of 463 ± 2.7 nM (n = 19 cells; $P = 0.00036$ vs. treatment start time at 2 m 30 s). This G-1–induced Ca^{2+} response was very similar in ERα siRNA transfected cells (471 ± 3.6 nM; n = 14 cells; $P = 0.00043$ vs. treatment start time at 2 m 30 s), but was almost completely abrogated in GPR30 siRNA–transfected cells (58 ± 1.3 nM; n = 19 cells; $P = 0.62$ vs. treatment start time at 5 m). Therefore, GPR30 mediated the G-1–induced Ca^{2+} mobilization.

Next, whether ERα and/or GPR30 mediate E_2–induced Ca^{2+} mobilization was investigated (Fig. 5B). In non-targeting siRNA–transfected cells, 10^{-6} M E_2 induced an increase in $[Ca^{2+}]_i$ of 159 ± 1.6 nM (n = 9 cells, $P = 0.0075$ vs. treatment start time at 4 m). However, in ERα siRNA–transfected cells, E_2 caused a greater increase in intracellular Ca^{2+} levels of 314 ± 3.2 (n = 17 cells; $P = 0.00058$ vs. treatment start time at 2 m 30 s), or almost a 2-fold the rise in $[Ca^{2+}]_i$ compared to the non-targeting siRNA–transfected cells. In GPR30 siRNA–transfected cells, the E_2–mediated Ca^{2+} response was blocked as E_2 administration resulted in only a non-significant rise in $[Ca^{2+}]_i$ of 52 ± 0.8 nM (n = 11 cells; $P = 0.35$ vs. treatment start time at 5 m). Immunoblot analysis indicated that the ERα siRNA effectively depleted ERα protein expression by 83%, and real-time qPCR analysis showed that the GPR30 siRNA effectively depleted GPR30 mRNA expression by 60 % (Fig. 5C). However, GPR30 mRNA levels were increased by 73% in ERα–depleted cells. The finding that ERα depletion in MCF-7 cells led to increased GPR30 expression, or likely de-repression of GPR30, is consistent with the prior finding that E_2 repressed GPR30 expression. Therefore, GPR30 and not ERα mediated the E_2–induced Ca^{2+} response. Further, depleting ERα led to increased E_2–induced Ca^{2+} mobilization likely due to de-repression of GPR30 levels.

GPR30 depletion promoted while the GPR30 agonist G-1 inhibited E_2–stimulated growth

The role of GPR30 in E_2–stimulated cellular proliferation was first examined by depleting GPR30 using siRNA methodology. To examine the effect of GPR30 depletion on growth, MCF-7 cells were transfected (as detailed under Methods) with a non-targeting siRNA pool and GPR30 (#8) siRNA, and then allowed to grow for 5 days in the absence (vehicle only control treatment) or presence of 10^{-9} M E_2 (Fig. 6A). To assess for potential differences in the number of cells seeded between the independently transfected non-targeting and GPR30 siRNA groups, additional cells were collected at day 0 when E_2 treatments were started. Proliferation was evaluated as cellular DNA mass per well, which was quantified using a fluorescent DNA–binding dye. Comparison of DNA mass between the control–treated cells in the non-targeting and GPR30 siRNA groups at day 0 indicated an equivalent number of cells were seeded for both groups. Likewise, comparison of the control–treated non-targeting and GPR30 siRNA groups at day 5 indicated that GPR30 depletion did not affect basal non-stimulated growth. However, GPR30 depletion potentiated E_2–stimulated growth by 2.1-fold ($P < 0.0001$), since in the non-targeting siRNA–transfected cells, E_2 treatment caused an increase of only 9.1 µg DNA/well compared to control treatment, but a greater increase of 19.2 µg DNA/well compared to control treatment in the GPR30 siRNA–transfected group. Analysis of the non-targeting and GPR30 siRNA–transfected cells for the level of depletion of GPR30 RNA levels by real-time PCR indicated a 75 % decrease between the respective control treatment groups and a 65 % decrease between the respective E_2 treatment groups (Fig. 6B). This real-time PCR analysis again showed that E_2 treatment reduced GPR30

RNA expression by 79 % and 70 % in non-targeting and GPR30 siRNA–transfected groups, respectively, compared to control–treated cells.

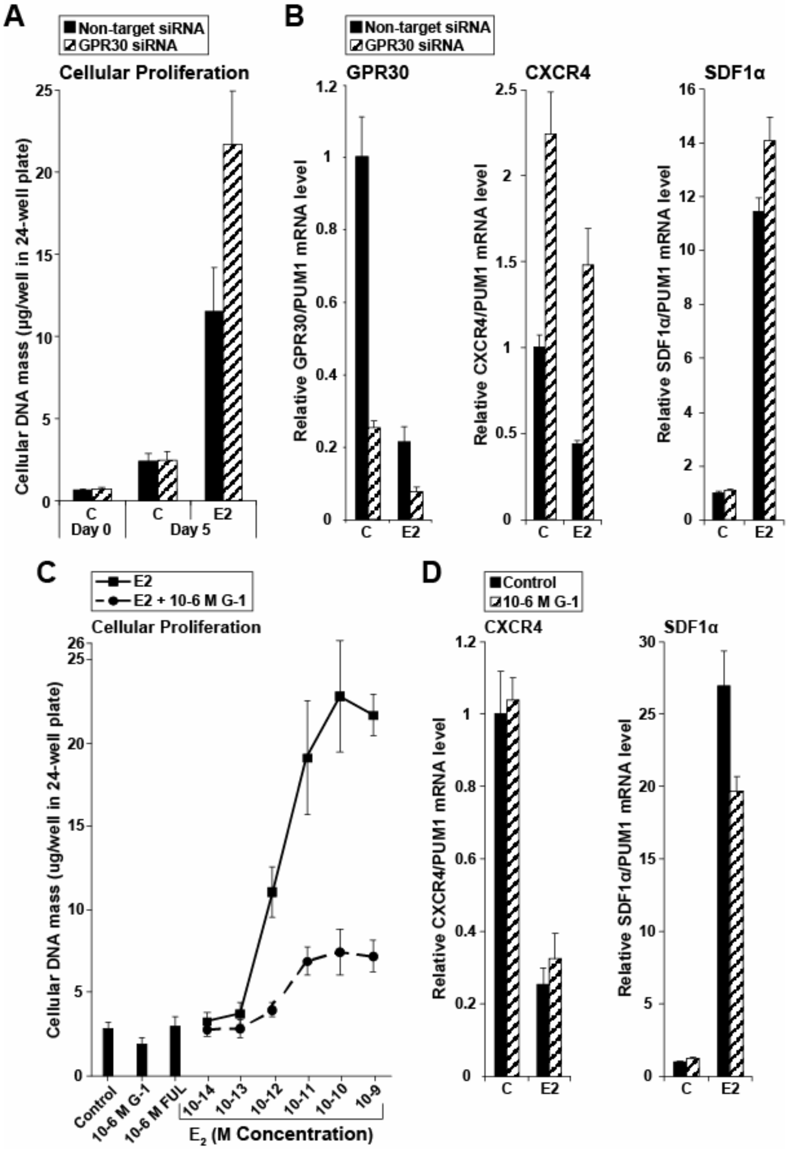

Figure 6. GPR30 inhibition promotes, while GPR30 activation blocks E_2-stimulated proliferation of MCF-7 cells. (A) E_2-stimulated growth in MCF-7 cells transfected with non-targeting pool siRNA (black bars) and GPR30 (#8) siRNA (hatched bars). Cells were transfected twice on consecutive days as described in Methods, and then seeded at 15,000 cells per well in 24-well dishes. Cells were collected on the day after seeding (day 0) and after 5 days of control (C) or 10^{-9} M E_2 treatment (E2). Proliferation was assessed as cellular DNA mass (µg/well). Data shown are the average of 24 wells and error bars the associated SDs. GPR30 depletion resulted in a 2.1-fold potentiation of E_2-stimulated growth. **(B)** GPR30, CXCR4 and SDF1α mRNA levels were determined by qPCR in non-targeting siRNA and GPR30 siRNA-transfected cells. Expression analysis was conducted following 24 h of 10^{-9} M E_2 or control treatment, and 48 h following the second transfection. GPR30 was effectively depleted. CXCR4 and E2-induced SDF1α mRNA levels were up-regulated in GPR30 siRNA- compared to non-targeting siRNA-transfected cells. **(C)** Proliferation of MCF-7 cells treated with a serial dilution series of E_2 in the absence and presence of 10^{-6} M G-1. G-1 blocked E_2-stimulated growth. **(D)** CXCR4 and SDF1α mRNA levels were determined by qPCR after 24 h control (C) or 10^{-9} M E_2 treatment. G-1 did not significantly affect CXCR4 mRNA expression but did reduce E2-induced SDF1α mRNA expression.

To begin to explore why GPR30 depletion promoted E_2–stimulated growth, changes in expression of key genes were measured by qPCR in these siRNA–transfected cells (Fig. 6B). To choose which genes should be investigated, correlations in gene expression were sought between GPR30 mRNA expression and all other genes in the NKI 295 breast cancer microarray data set. Of the GPR30 correlating genes found, another seven-transmembrane-domain G protein-coupled receptor CXCR4 was chosen for investigation since it showed a significant inverse relationship (Pearson's correlation coefficient $\rho = -0.274$, adjusted for all other gene comparisons $P = 0.00008$), and both GPR30 (13, 15) and CXCR4 (16) promote migration of breast cancer cells. In non-targeting siRNA–transfected cells, E_2 down-regulated CXCR4 mRNA by 57 % compared to control as previously observed (17). However, in GPR30 siRNA–transfected cells, the basal level of CXCR4 expression under control treatment conditions was 124 % higher than in non-targeting siRNA transfected cells. Although in GPR30 siRNA–transfected cells, E_2 still down-regulated CXCR4 expression by 34% compared to control treatment, but CXCR4 mRNA levels remained elevated by 236 % in E_2–treated GPR30 siRNA–transfected cells relative to E_2–treated non-targeting siRNA–transfected cells. The primary ligand of CXCR4 is stromal cell-derived factor 1 alpha (SDF1α, also termed CXCL12), and SDF1α expression is an E_2–inducible gene (18-20) that acts as a potent mitogen of breast cancer cells including MCF-7 (18), hence expression of this gene was also investigated. In non-targeting siRNA–transfected cells, E_2 induced SDF1α expression 11.4-fold compared to control treatment, but in GPR30 siRNA–transfected cells, SDF1α was induced by E_2 to a higher level of 14.0-fold ($P = 0.0044$). Therefore, depletion of GPR30 enhanced E_2–stimulated growth, possibly due to increased CXCR4 and SDF1α mRNA expression.

To complement the proliferation experiments involving GPR30 depletion, the effect of activating GPR30 using 10^{-6} M G-1 on growth was also examined (Fig. 6C). MCF-7 cells were treated with increasing concentrations of E_2 in the absence and presence of 10^{-6} M G-1 and allowed to grow for 6 days. As expected, MCF-7 cells showed a growth stimulatory response to E_2 in a concentration dependent manner with 8.1-fold maximal growth occurring at 10^{-10} M E_2 compared to control treatment. G-1 inhibited basal (control treatment) growth by 32% ($P < 0.0001$). G-1 also blocked E_2–stimulated growth (all E_2 treatment groups vs. paired E_2 + G-1 treatment groups, $P = 0.0001$, 1-way ANOVA), and in particular, G-1 inhibited 10^{-10} M E_2–stimulated growth by 68 % relative to 10^{-10} M E_2 alone ($P < 0.0001$). Again CXCR4 and SDF1α mRNA levels were examined (Fig. 6D). No significant changes in CXCR4 mRNA expression were observed. E_2 induced SDF1α mRNA expression 26.9-fold compared to control treatment, whereas in the presence of G-1, E_2 induced SDF1α only 19.7-fold ($P = 0.0087$). Therefore, G-1 profoundly inhibited E_2–stimulated growth, and this growth inhibition may have involved an attenuated induction of SDF1α mRNA expression.

G-1–activated GPR30 blocks cell cycle progression at G(1)-phase

To further explore inhibition of E_2–stimulated growth by G-1, the effect of G-1 on cell cycle progression was investigated. MCF-7 cells were synchronized as described in the Methods, and then treated with appropriate combinations of 10^{-9} M E_2 and 10^{-6} M G-1 for 24 h, followed by staining for DNA content using propidium iodide and analysis by flow cytometry (Fig. 7A). As expected, E_2 treatment led to a decrease in the proportion of cells in G(1)-phase of the cell cycle from 69.8 % under control conditions to 42.7 % under E_2 conditions, and a concomitant increase in the percentage of cells in S-phase from 19.8% in control–treated cells to 37.3% in E_2–treated cells. Treatment with the GPR30 agonist G-1 did not significantly change the proportion of cells in G(1)-phase of the cell cycle, but did significantly decrease S-phase cells from 19.8 % under control to 14.7 % under G-1 treatment conditions ($P < 0.0001$). Importantly, the addition of G-1 to E_2 led to retention of an additional 11.6 % of the cells in G(1)-phase of the cell cycle (42.7 % in E_2 vs. 54.4 % in E_2 + G-1–treated cells, $P < 0.0001$), and prevented 13.2 % of cells from entering S-phase (37.7 % in E_2 vs. 24.5 % in E_2 + G-1–treated cells, $P < 0.0001$). G-1 did not significantly alter the proportion of G(2)/M-phase cells in the absence or presence of E_2. Therefore, G-1 blocked E_2–stimulated cells from cell cycle progression at G(1)-phase of the cell cycle.

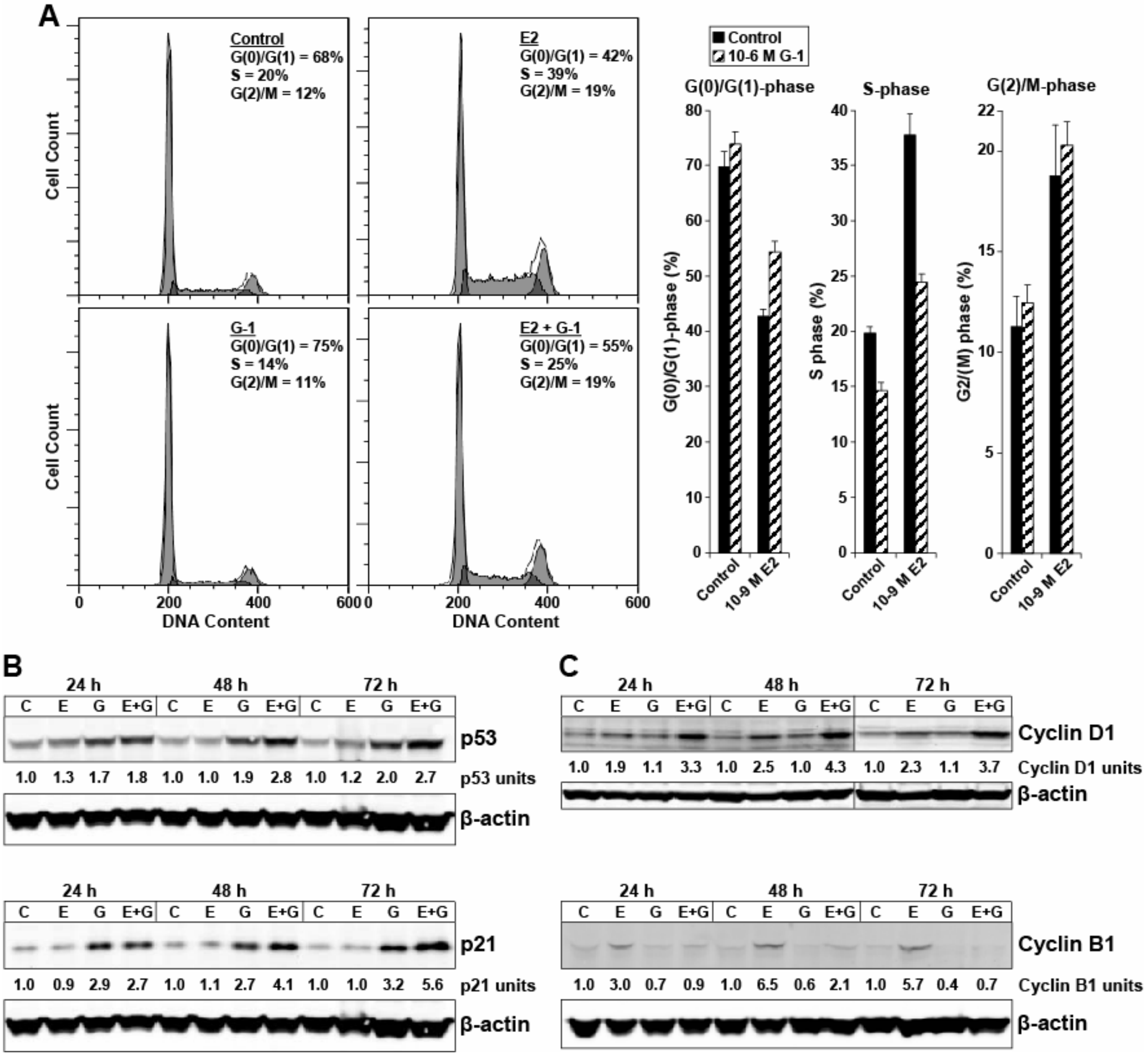

Figure 7. G-1 inhibits cell cycle progression in E_2-stimulated MCF-7 cells by producing a block at G(1)-phase. **(A)** Cell cycle distribution as determined by propidium iodide staining of DNA content and flow cytometry. Cells were synchronized as described in Methods, and then treated as indicated for 24 h. Thirty-thousand cells per sample were collected. Data were analyzed using FloJo v7.2.5. Representative histograms are shown. Bars represent the average of 3 replicates and error bars their associated SDs. G-1 led to an increase in the percentage of E_2-stimulated cells in G(1)-phase, and a decrease in the percentage of E_2-stimulated cells in S-phase, compared to E_2 alone treatment. E_2 and G-1 regulation of protein levels of **(B)** the cell cycle regulators p53 and p21(Waf1/Cip1), and of **(C)** the cyclins D1 and B1. MCF-7 cells were control (C), 10^{-9} M E_2 (E2) and 10^{-6} M G-1 (G) –treated as indicated for 24, 48, and 72 h. Protein levels were analyzed by immunobloting using a Li-Cor Odyssey infrared scanner. Quantitation of protein levels normalized to β-actin are indicated. G-1 alone and G-1 added to E_2 promoted p53, p21, and cyclin D1 accumulation, while preventing cyclin B1 accumulation.

The pathway by which G-1 induced a cell cycle block was investigated by measuring protein expression of key cell cycle regulators including the tumor suppressor p53 and the cyclin-dependent kinase inhibitor p21(Waf1/Cip1) (Fig. 7B), as well as the G(1)-phase–specific cyclin D1 and G(2)/M-phase–specific cyclin B1 (Fig. 7C). MCF-7 cells were treated with combinations of 10^{-9} M E_2 and 10^{-6}

M G-1, and then collected at 24 h, 48 h, and 72 h followed by semi-quantitative immunoblot analysis. Both p53 and p21 proteins were up-regulated in G-1 and E_2 + G-1–treated cells compared to control–treated cells, with a maximal 2.7-fold induction of p53 and a 5.6-fold induction of p21 occurring at 72 h in E_2 + G-1–treated cells (Fig. 7B). Induction of p53 and p21 protein levels would be consistent with promoting a cell cycle block. E_2 up-regulated both cyclins D1 and B1 across the time course compared to control treatment, whereas G-1 alone did not induce either of these cyclins (Fig. 7C). However, the addition of G-1 to E_2 potentiated the up-regulation of cyclin D1 at each time point compared to E_2 alone; for instance E_2 alone induced cyclin D1 2.5-fold relative to control–treated cells, but in E_2 + G-1–treated cells, cyclin D1 was induced 4.3-fold. In contrast, the addition of G-1 to E_2 prevented accumulation of cyclin B1 throughout the time course compared to E_2 alone; for example E_2 induced cyclin B1 5.7-fold at 72 h, but this was completely blocked in E_2 + G-1–treated cells and instead there was a 30 % reduction compared to control–treated cells. Since cyclin D1 is induced during G(1)-phase and degraded in S-phase (21, 22), whereas cyclin B1 accumulates during G(2)-phase and degrades upon M-phase entry (23), these data are consistent with G-1 blocking cell cycle progression in G(1)-phase of the cell cycle before cyclin D1 degradation occurred and before cylin B1 accumulated.

The mechanism by which G-1 inhibited growth likely involves the large ~460 – 470 nM increases in cytosolic Ca^{2+} concentrations (Fig. 4B). Intracellular Ca^{2+} signaling is a critical regulator of numerous cellular activities including proliferation [reviewed in (24)] and cell cycle progression [reviewed in (25, 26)]. Transient $[Ca^{2+}]_i$ increases occur when emerging from quiescence, at the G(1)/S border, during S-phase, and upon exit from mitosis. Transient $[Ca^{2+}]_i$ increases activate calmodulin and calcium/calmodulin-dependent kinases (CaM-Ks) I and II to regulate cell cycle progression (25). Cam-K II acts at the G(1)/S border, progression from G(2) to M-phase, and the metaphase/anaphase transition (26). Cam-K I is also involved in cell cycle regulation as its inhibition leads to cell cycle arrest in MCF-7 cells at the G(1)-phase of the cell cycle (27). The transient $[Ca^{2+}]_i$ increases also activate Ca^{2+}-dependent proteases such as calpain that are important in cleavage of some cyclins (26). However, aberrant increases in intracellular Ca^{2+} levels can lead to inhibition of proliferation, and induce apoptosis [reviewed in (28). For example, the plasma membrane Ca^{2+}-ATPase (PMCA) pumps Ca^{2+} across the plasma membrane out of the cell to lower cytosolic Ca^{2+} levels after transient Ca^{2+} increases. Partial inhibition of PMCA in MCF-7 cells causes a moderate increase in intracellular Ca^{2+} levels which leads to inhibition of proliferation by altering cell cycle kinetics (29). As another example, extracellular ATP produces a transient but large increase in $[Ca^{2+}]_i$ through binding of purinergic receptors in MCF-7 cells that leads to growth inhibition and apoptosis (30). Compounds that directly mobilize Ca^{2+} through the store operated calcium entry mechanism (SOCE) such as thapsigargin, an inhibitor of the sarco/endoplasmic reticulum Ca^{2+}–ATPase pump, induce apoptosis (31), and the mechanism of action of numerous anti-tumor drugs (32), such as β-lapachone (33), involve increases in $[Ca^{2+}]_i$.

Taken together, GPR30 inhibits growth of ERα–positive breast cancer. Our studies also indicate that pharmacologic activation of GPR30 shows promise in combating E_2–responsive breast cancer. Hence, G-1 may represent the first in a new class of drugs for use alone or in conjunction with conventional antihormonal therapeutics in breast cancer.

KEY FINDINGS

- GPR30 was overexpressed and exhibited parallel increases in functional activity in estrogen deprivation-resistant MCF-7/5C and MCF-7/2A cells compared to wild-type MCF-7 cells (Fig. 1).
- Mining of the breast carcinoma microarray data representing 1,250 specimens across 5 independent cohort showed increased GPR30 expression associated with ERα–positive status (Fig. 2).

- E_2 decreased both ERα and GPR30 mRNA levels, but the GPR30 specific agonist G-1 did not, indicating that E_2's effect on ERα and GPR30 expression was mediated by ER (Fig. 3).

- RNA interference-mediated depletion of GPR30 blocked E_2– and G-1–induced Ca^{2+} mobilization, but ERα depletion did not. Instead, ERα knockdown augmented the E_2-induced Ca^{2+} response; likely due to ERα depletion leading to increased GPR30 expression (Fig. 5).

- In proliferation studies, GPR30 knockdown promoted, whereas G-1 profoundly inhibited E_2-stimulated growth of MCF-7 cells. Consistent with increased growth in GPR30-depleted cells, CXCR4 and E_2-induced SDF1α expression were increased, while in G-1–treated and growth inhibited cells, E_2-induced SDF1α expression was reduced but CXCR4 expression was unaffected (Fig. 6).

- Flow cytometry showed that G-1 prevented E_2-stimulated cells from entering S phase. Concurrently, p53, p21, and G(1)-phase specific cyclin D1 accumulated, while the G(2)/M-phase specific cyclin B1 did not accumulate further supporting a G(1)-phase cell cycle block (Fig. 7).

Future work on GPR30 will focus on its role in modulating growth and E_2-induced apoptosis in estrogen deprivation-resistant MCF-7:5C and MCF-7:2A cells.

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TASK 2. FCCC/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2b-2: (Ariazi and Jordan) – To confirm and validate developing pathways of E_2-induced breast cancer cell survival and apoptosis.

Task 2b is organized into sub-sections 1-4 according to projects led by senior investigators in Dr. Jordan's laboratory that involve deciphering pathways of E_2-induced breast cancer cell survival and apoptosis.

Here we report work completed on Task 2b-2 at the Fox Chase Cancer Center (FCCC) site during year 3 of this COE.

Studies carried out at FCCC by Eric Ariazi PhD, in the laboratory of Dr. Jordan.

INVOLVEMENT OF UNFOLDED PROTEIN STRESS AND CASPASE-4 (CASP4) IN E_2-INDUCED APOPTOSIS IN ESTROGEN DEPRIVATION-RESISTANT MCF-7 BREAST CANCER CELLS

Introduction

To identify potential mechanisms of E_2-induced apoptosis, we have conducted global gene expression profiling by microarray experiments of two *in vitro* models of long-term estrogen deprivation-resistance developed in our laboratory, MCF-7:2A (or 2A) and MCF-7:5C (or 5C) cells, compared to wild-type estrogen-responsive MCF-7:WS8 (or WS8) cells. Bioinformatic analysis of these gene expression microarray data is described in detail under Task 4. Here we describe functional validation of a potential mechanism of E_2-induced apoptosis that was indicated by the bioinformatic analysis. This mechanism involves E_2 producing unfolded protein stress, which leads to induction and activation of endoplasmic reticulum stress-associated caspase-4 (CASP4). A causal role of CASP4 was validated by showing that CASP4 inhibition using Z-LEVD-FMK completely reversed E_2-inhibited growth of 5C cells and blocked E_2-induced morphologic changes associated with apoptosis in 5C cells.

WORK ACCOMPLISHED

Cell Line Characterization

To assist in relating gene expression profiles to the phenotypes of WS8, 2A and 5C cells, growth of these cell lines in response to E_2 treatment was examined (Fig. 1). Cells were seeded in multi-well plates and allowed to grow in the presence or absence of E_2 over 7 days (WS8 and 5C cells) or 12 days (2A cells). DNA mass per well was measured daily using a DNA-binding fluorescent dye (Hoechst 33258). The wild-type MCF-7:WS8 cells exhibited a 6.8-fold increase in growth after 7 days of 10^{-9} M E_2 treatment compared to control (no E_2) treatment (Fig. 1A). The resistant MCF-7:2A cells grew robustly in the absence of E_2 over 12 days. E_2 did not affect growth of the 2A cells over the first 6 days, but did inhibit growth beginning at day 7 through day 12, such that at day 12, E_2 blocked growth by 62.5% (Fig. 1B). Hence, the 2A cells exhibited an initial phase of E_2-independent growth followed by a second phase of E_2-inhibited growth. The resistant 5C cells continually proliferated in the absence of E_2 over 7 days. The DNA mass per well of the 5C cells also increased in the presence of E_2, but only for the first 4 days. It is important to note that within this period of apparent growth, E_2 caused gross morphologic changes in the 5C cells such as rounding, blebbing and detachment from the plate by day 3 and subtle changes as early as day 2 (Fig. 11B shows the E_2-induced morphology after 4 days of E_2

treatment). In E_2-treated 5C cells, the DNA mass per well decreased steadily from day 4 to day 7 such that by day 7, there was a 105 % decrease in DNA mass per well compared to control treatment, or 5% less DNA then at day 1 of the experiment (Fig. 1C). Therefore, the 5C cells displayed a relatively rapid E_2-induced growth inhibitory response compared to the delayed growth inhibitory response in the 2A cells. We have previously reported that these growth inhibitory responses to E_2 reflect induction of apoptosis occurring with rapid kinetics in the 5C cells (1) and slow kinetics in the 2A cells (described in the Year 2 CoE Annual report).

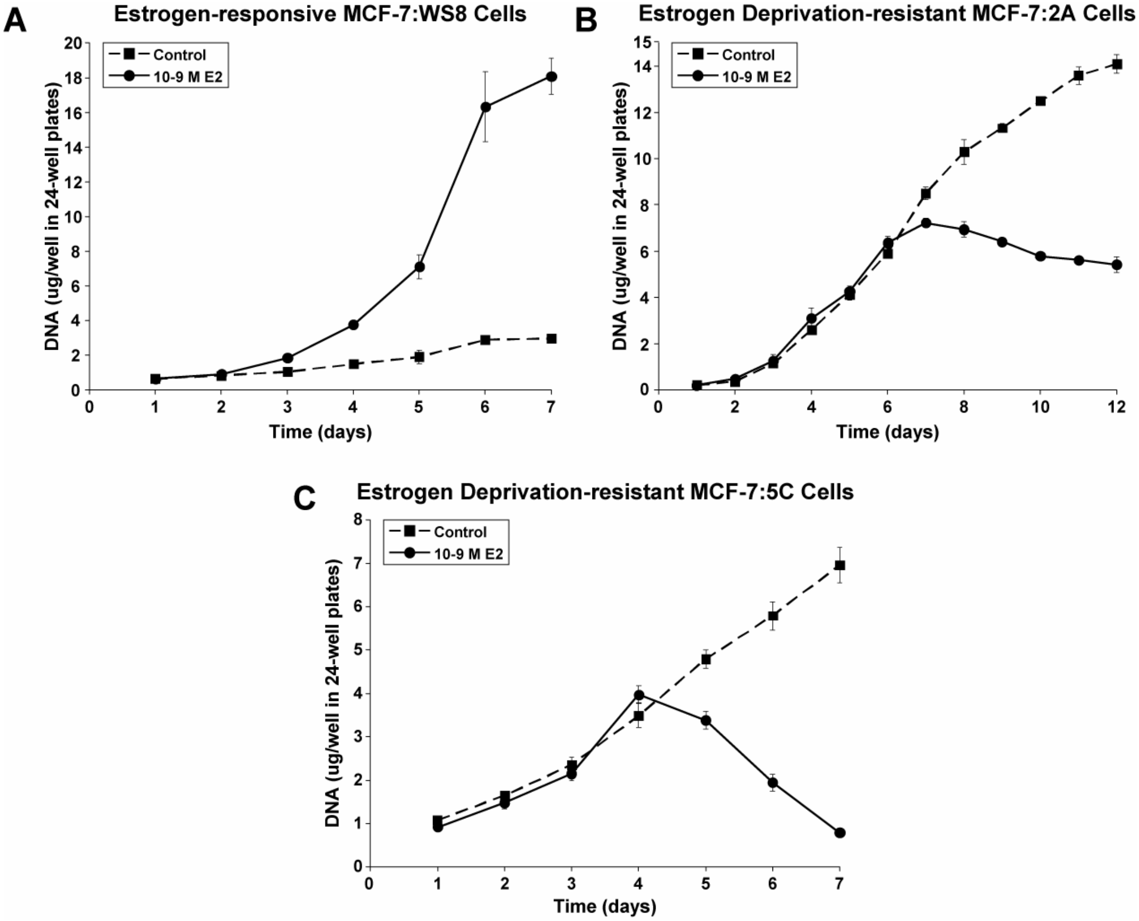


Figure 1. E_2-regulated Proliferation of Wild-type MCF-7:WS8 and of Estogen Deprivation-Resistant MCF-7:2A and MCF-7:5C cell lines.

We also characterized HER2 expression, ERα expression, and ER functional activity in the wild-type WS8 and resistant 2A and 5C cells (Fig. 2). HER2 protein levels were measured since its increased expression has been shown to associate with antihormone resistance in clinical breast carcinomas, and has been shown to be causally involved in the development of antihormone resistance in *in vitro* and *in vivo* model systems. HER2 and ERα protein levels were measured following 48 h of control or 10^{-9} M E_2 treatment by semi-quantitative immunoblot analysis. HER2 protein levels were decreased by approximately 4-fold and 5-fold in the 2A and 5C cells, respectively, compared to the WS8 cells (Fig. 2A). Therefore HER2 was unlikely to have contributed to the development of resistance to estrogen deprivation in these cells. In contrast, ERα protein levels were 5-fold and 1.9-fold increased in the 2A and 5C cells, respectively, compared to the WS8 cells (Fig. 2A). It is likely that derivation of the 2A and 5C cells under estrogen-free conditions selected for increased ERα expression since this would translate into an increase in unliganded ER activity. This hypothesis was evaluated by characterizing ERE (estrogen responsive element)-dependent transcriptional activity using a previously described dual-luciferase reporter gene assay (2). Cells were transiently transfected for 24 h with an ERE(5x)-TATA box driven firefly luciferase reporter plasmid, and for normalization of transfection efficiency, co-transfected with a basal TATA box-regulated renilla luciferase reporter plasmid. Following the transfection, the cells were treated as indicated in Fig. 2B-C for 24 h. The ERE-dependent transcriptional activity is presented in two plots, in one plot as fold-induction relative to control-treated WS8 cells (Fig. 2B), and in a second plot as fold-induction relative to control treatment levels in each cell line independently (Fig. 2C). When examining ERE activity in the resistant cells relative to control-treated WS8 cells (Fig. 2B), the basal (control treatment) ERE-dependent transcriptional activity was 4.8-fold higher in 2A cells $P < 0.0001$), and 66 % higher in 5C cells ($P = 0.001$) than in WS8 cells. Hence, the unliganded ER transcriptional activity was increased in the 2A and 5C cells and correlated with the ERα levels in the cell lines. E_2 maximally stimulated ERE activity to a higher level in 2A cells (22.5-fold) but to a lower level in 5C cells (7.4-fold) compared to control-treated WS8 cells (maximum E_2-stimulated activity = 15.4-fold). However, when taking into account the already elevated basal ERE activity, the ability of E_2 to maximally induce ERE activity was reduced in the resistant cells versus the wild-type cells: E_2 maximally induced ERE-dependent activity by only 3.9-fold in 10^{-8} M E_2-treated compared to control-treated 2A cells, and by 4.4-fold in 10^{-8} M E_2-treated compared to control-treated 5C cells, whereas E_2 maximally stimulated ERE activity by 15.4-fold in 10^{-9} M E_2-treated compared to control-treated WS8 cells (Fig. 2C). Therefore, the basal unliganded ER transcriptional activity was increased in the resistant cells versus the wild-type cells, resulting in a relatively lower fold-induction of activity upon E_2 stimulation.

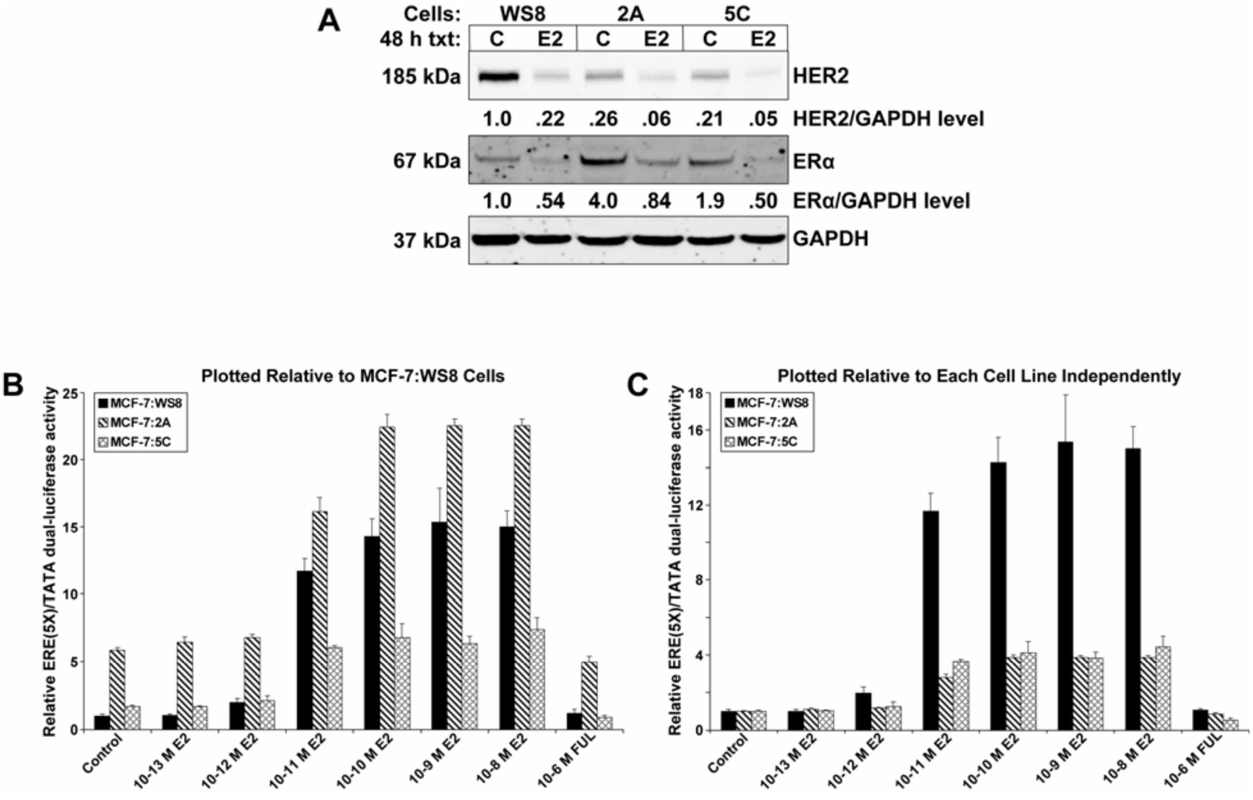


Figure 2. HER2 and ERα status in the wild-type and resistant cell lines. (A) Decreased HER2 and increased ERα in resistant Cells compared to WS8 cells. (B) ERE-dependent transcriptional activity.

Gene Expression Profiling

Gene expression profiling of the wild-type WS8 and estrogen deprivation-resistant 2A and 5C cells was carried out using 2-color Agilent 4x44k human oligonucleotide microarrays. Microarrays were competitively hybridized with RNA isolated from 10^{-9} M E_2-treated cells against matched control-treated cells over a 96 h time course, which corresponds to the time scale in which 5C cells undergo E_2-induced apoptosis whereas the 2A cells exhibit E_2 independence. 2A cells were additionally characterized by microarrays over a long-term time course of 3 to 9 days to capture their delayed E_2 apoptotic response. Task 4 describes analyses of these gene expression microarray experiments.

Caspases

Using Gene Set Enrichment Analysis (GSEA) (3), a set of co-regulated genes involved in apoptosis was identified in 5C cells treated with E_2 for 96 h (see Task 4b/FCCC.) In this set of apoptotic genes, multiple caspases were induced by E_2 in the resistant cells and not in the wild-type cells. The profiles of these caspases were extracted from the automated graphing microarray database (described under Task 4a: FCCC/Ariazi and Jordan) and are shown in Fig. 3. These caspases were (ordered first by time and second by magnitude): CASP4, CASP5, CASP10, CASP1, and CASP8. The earliest caspases induced, CASP4 and CASP5, were significantly increased by 12 h (adjusted $P = 0.0003$ and $P = 0.0014$, respectively) in 5C cells. However CASP4 attained a higher level of induction (3.8-fold) than CASP5 (2.6-fold) by day 4 of E_2 treatment in 5C cells. CASP4 and CASP5 were also the only caspases that were E_2-induced in the 2A cells during the long-term time course. Interestingly, human CASP4 and CASP5 are highly related at the amino acid level to mouse CASP12 (4), which is specifically involved in an endoplasmic reticulum stress-induced apoptosis pathway (5). Likewise, human CASP4 has been demonstrated to be specifically localized to the endoplasmic reticulum, cleaved when cells are treated with endoplasmic stress-inducing agents but not other apoptotic agents, and its depletion by siRNA can prevent endoplasmic stress-induced apoptosis (4). Therefore it has been suggested that CASP4 is vital for endoplasmic reticulum stress-induced apoptosis.

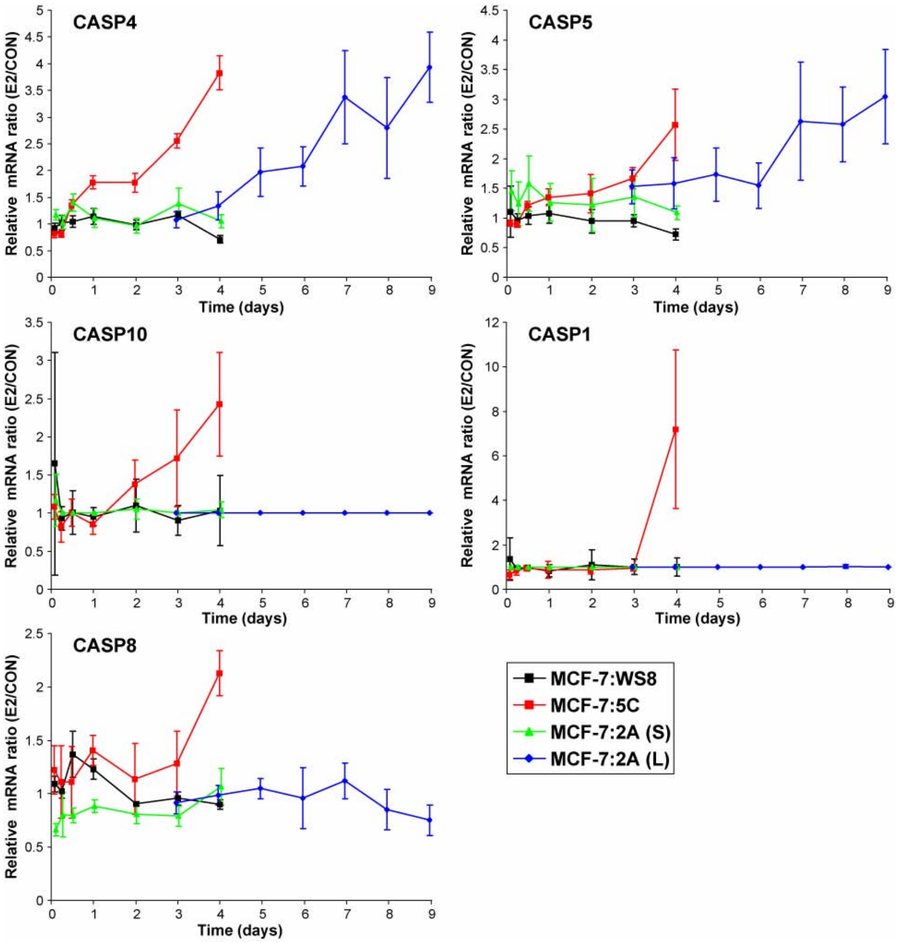


Figure 3. E_2-induced Caspase mRNA Expression in Resistant Cells by Microarrays.

Stress Response Genes

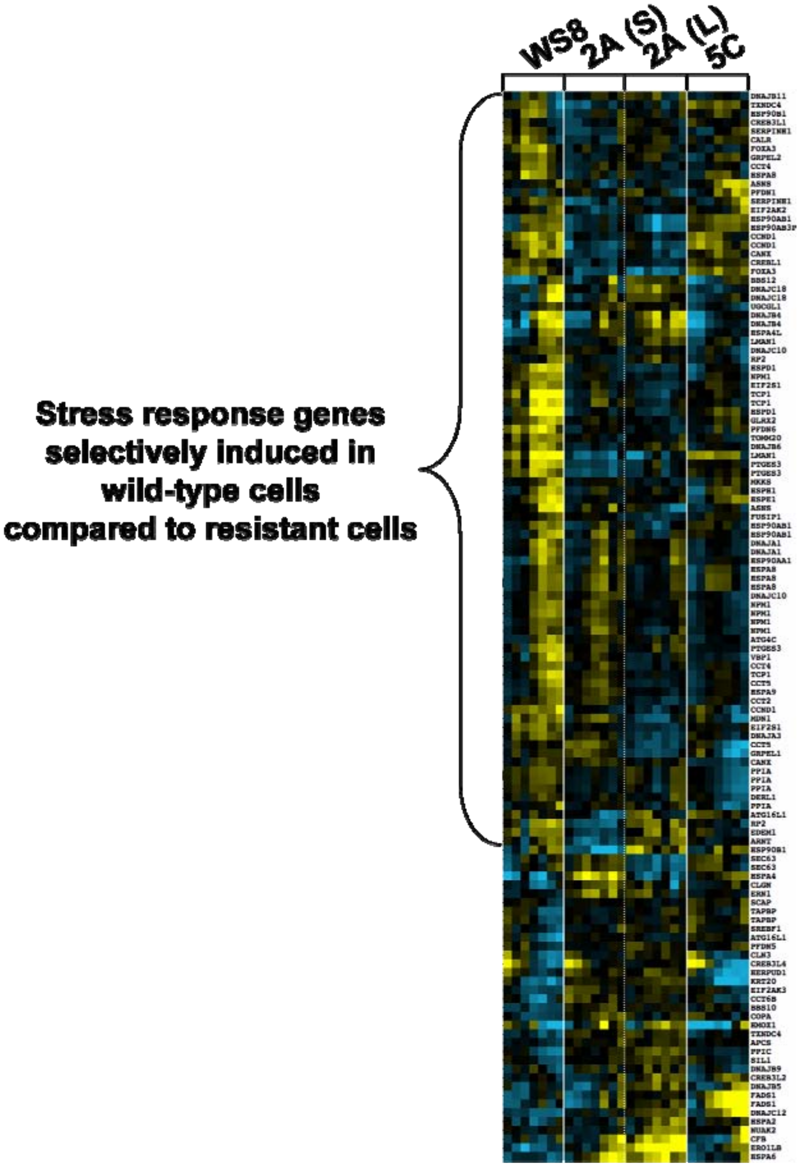


Figure 4. GO analysis identified 122 Stress Response Genes Differentially Regulated by E_2 in Estrogen Deprivation-resistant MCF-7:2A and MCF-7:5C Cells Compared to wild-type MCF-7:WS8 Cells.

In addition to finding a set of apoptosis genes enriched in E_2-treated resistant cells, an analysis of gene ontology (GO) terms found genes involved in cellular response to stress were statistically over-represented (P = 0.001), including those involved in endoplasmic reticulum stress (Fig. 4). This is consistent with a potentially important role for CASP4 in E_2-induced apoptosis. Protein synthesis and folding of secreted, membrane bound, and some organelle-target proteins occurs in the endoplasmic reticulum. To promote optimal protein folding, important enzymatic steps and molecular chaperones involved in this process require several factors such as ATP, calcium (Ca^{2}+) and an oxidizing environment. Protein folding often occurs in an ATP-dependent manner, many chaperones require Ca^{2+} as a bound cofactor, and formation of disulfide bonds requires and an oxidizing environment. When cellular stresses perturb energy levels, the redox state, or the Ca^{2+} concentration, accumulation of unfolded proteins and protein aggregation occurs; this condition is referred to as endoplasmic reticulum stress. To relieve endoplasmic reticulum stress, an unfolded protein response (UPR) is triggered to clear the unfolded proteins and export them to the cytosol for degradation. The UPR is initiated by a key Ca^{2+}-dependent chaperone termed BiP (also termed GRP78 for glucose-regulated protein, 78 kDa; and HSPA5 a member of the HSP70 family). BiP not only binds unfolded proteins, but also binds the luminal domains of endoplasmic reticulum transmembrane receptors, preventing their oligomerization. When unfolded proteins accumulate, BiP is released from binding the transmembrane receptors, allowing them to oligomerize and autophosphorylate to initiate a UPR signal. Although some unfolded proteins may also directly bind and activate the transmembrane receptors. The critical endoplasmic reticulum transmembrane receptors include PERK (protein kinase-like endoplasmic reticulum kinase, also known as EIF2AK3), IRE1 (inositol-requiring kinase, also known as ERN1) and activating transcription factor 6 (ATF6). The UPR signals to attenuate protein translation, induce expression of additional chaperones, and export malfolded proteins to the cytosol for ubiquitylation and proteasome-mediated degradation. If the UPR fails to relieve the stress, the function of the UPR switches from promoting cell survival to promoting cell death. Thus, excessive or prolonged endoplasmic reticulum stress typically induces apoptosis, but can also induce autophagy, a catabolic process that engages lysosomes to degrade insoluble protein aggregates which can not be eliminated by the proteasome [reviewed in (6)].

Since in both the 5C and 2A cells, E_2 induced CASP4, which is associated with endoplasmic reticulum stress-induced apoptosis, and the GO analysis identified stress response genes, the microarray gene expression database was interrogated for genes involved in endoplasmic reticulum stress and associated factors. Examples of the identified stress response genes are shown in Figs. 5-9, although more genes than shown in these figures were found.

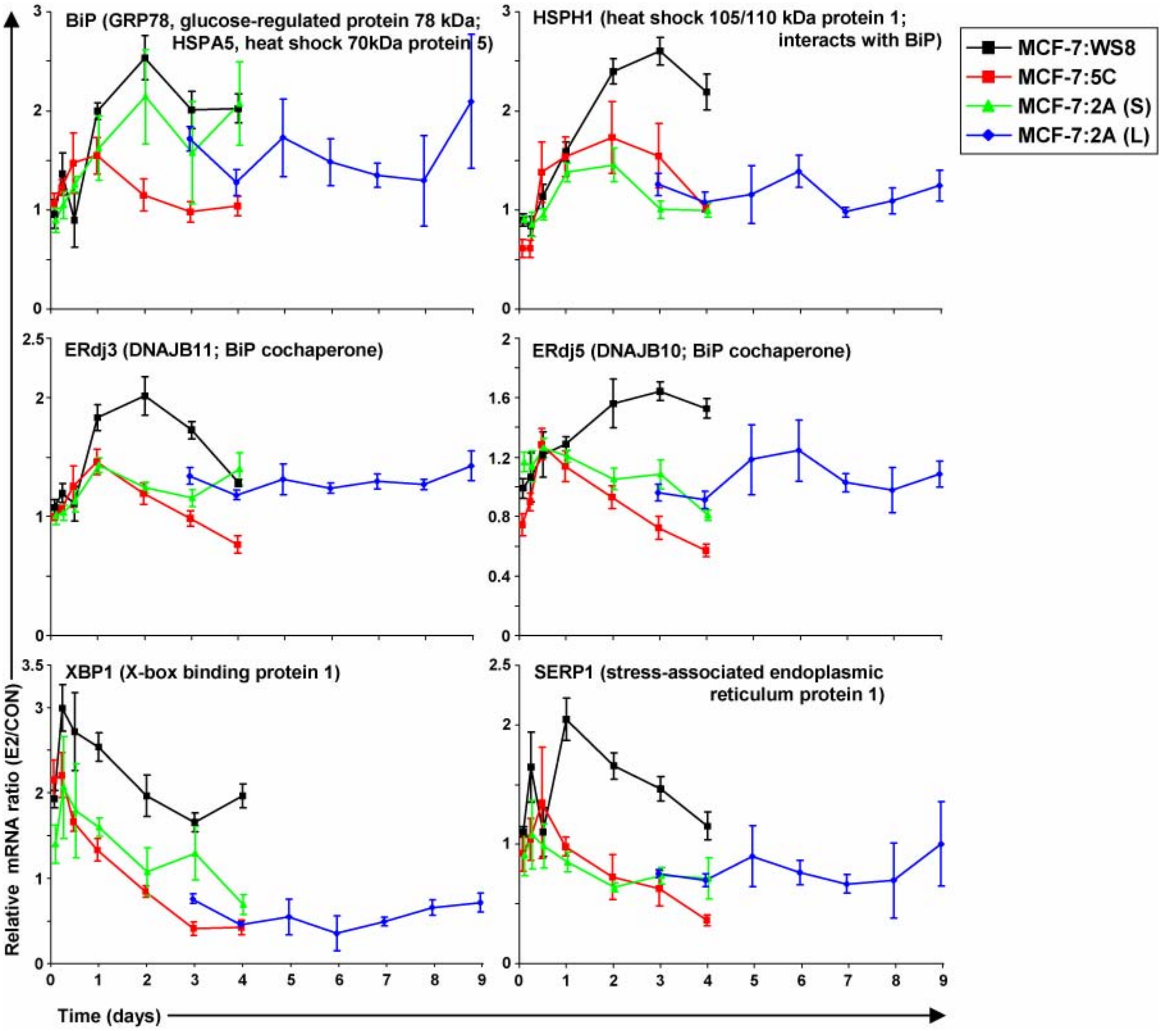


Figure 5. Decreased Induction of Genes Involved in Endoplasmic Reticulum Stress Response.

Fig. 5 shows BiP, and several BiP cochaperones (HSPH1, ERdj3, and ERdj5) were selectively induced in wild-type WS8 cells compared to resistant 5C and 2A cells. However, BiP showed an intermediary response in 2A cells, and hence may help explain the delayed kinetics of E_2-induced apoptosis in 2A cells. However, the lack of induction of the BiP cochaperones ERdj3 and ERdj5, and the BiP binding protein HSPH1 in both resistant cell lines would attenuate BiP activity in the 2A cells. XBP1 (X-box binding protein 1) and SERP1 (stress-associated endoplasmic reticulum protein 1) showed a similar decreased induction by E_2 in the resistant cells. XBP1 mRNA undergoes an unconventional splicing event mediated by the endonuclease activity of IRE1 to generate a short isoform of the protein XBP1(S) that is an activated transcription factor and binds the promoters of several genes involved in the UPR (6). SERP1 suppresses aggregation and degradation of membrane proteins in response to stress and facilitates their subsequent glycosylation upon release from stress (7). Thus, high expression of factors involved in protein folding in wild-type WS8 cells, and low expression in resistant 5C and 2A cells was observed repeatedly. This generalized gene expression pattern likely reflected E_2-induced proliferation in the WS8 cells which required increased protein translation. This would cause increases in unfolded proteins and hence a UPR that leads to induction of BiP, its cochaperones, XBP1 and SERP1 to accelerate protein folding and stabilize membrane proteins. However, the resistant cells were likely deficient in mounting a UPR, which would then result in aberrant accumulation of unfolded proteins.

In response to endoplasmic reticulum stress, Bcl2 family members, which directly regulate apoptosis, are targeted (6). The transmembrane receptor IRE1, through apoptosis signal-regulating kinase 1 (ASK1), signals to Jun N-terminal kinase (JNK) to phosphorylate and activate Bim (BCL2L11), a pro-apoptotic Bcl2 family member, while inhibiting anti-apoptotic Bcl2. Also, several signaling proteins activated by endoplasmic reticulum stress, including XBP1, converge on the promoter of the encoded transcription factor C/EBP homologous protein CHOP (also GADD153/DDIT3) to induce its expression. CHOP then transcriptionally induces Bim and simultaneously inhibits transcription of Bcl2. Anti-apoptotic Bcl2 was greatly induced by E_2 in wild-type cells but not in resistant 5C and 2A cells, whereas pro-apoptotic Bim was E_2-induced in the resistant cells but to a much lesser extent in wild-type WS8 cells (Fig. 6). The observed expression profiles of Bcl2 and Bim support that they were regulated by an endoplasmic reticulum stress-induced apoptotic pathway.

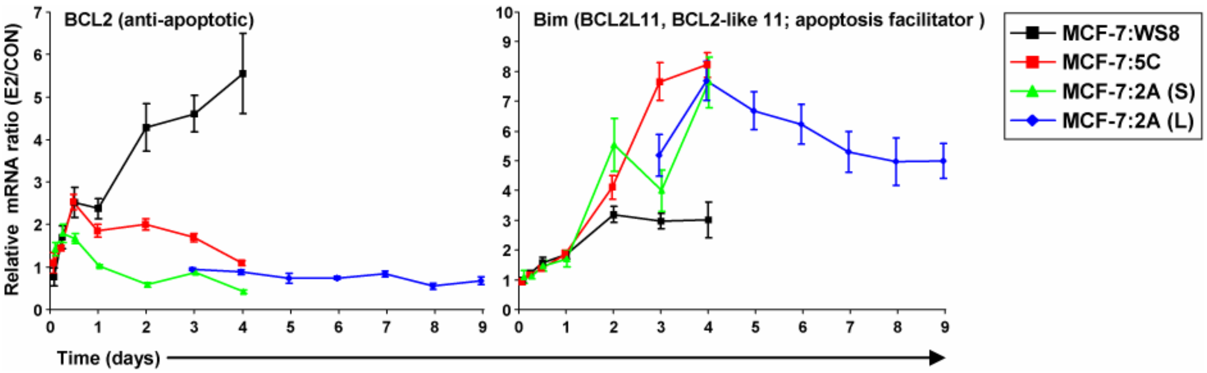


Figure 6. Differential Expression of Bcl2 Family Members Associated with Endoplasmic Reticulum Stress.

Not only did gene expression profiles suggest a UPR to due endoplasmic stress in resistant cells, but also a general deficiency in the ability to fold proteins. The pattern of E_2-mediated induction in wild-type cells of important protein folding genes, but a lack of their induction in resistant cells, was observed frequently. For instance, E_2 failed or partially failed to induce many chaperones in the heat shock superfamily, and their cochaperones (Fig. 7). The decreased induction of many heat shock factors in 5C and 2A cells may be related to a lack of induction of the transcription factor HSF2 (heat shock factor 2) and HSF2BP (heat shock factor 2 binding protein), which interacts with HSF2. HSF2 forms a heterotrimer with HSF1 (8) to bind the promoters of several heat shock factors including Hsp70 (9) and Hsp90 members (10). Thus Hsp70s and Hsp90s and their cochaperones, were deregulated to varying degrees in resistant cells compared to wild-type cells. For instance HSPA13, which is an HSP70 member, and DNAJC18, which as an Hsp40 that acts as an Hsp70 cochapererone by stimulating its ATPase activity (11), were robustly stimulated in WS8 but not in 5C or 2A cells. Hsp70s have a high affinity for unfolded protein when bound to ADP, stabilizing them and preventing them from aggregation until properly folded, then release folded protein upon binding ATP. (11). Interestingly, Hsp70s can directly inhibit apoptosis by binding Apaf1 in the apoptosome, preventing recruitment of procaspase-9 (12). HSP90AA1 and HSP90AB1 were E_2-induced in wild-type WS8 but not in resistant 5C cells, but did show partial to full induction in resistant 2A cells. However, their respective cochaperones AHA/AHSA1 (activator of heat shock 90 kDa protein ATPase homolog 1) (13) and HSP90 cochaperone p23 (also PTGES3, prostaglandin E synthase 3) (14), were significantly less induced in 2A cells (as well as in 5C cells), thereby attenuating HSP90 activity in these cells. Interestingly, Hsp90s show some selectivity in their substrate (or client) proteins for signaling proteins, and in particular, steroid hormone receptors such as ERα (13). In the unliganded state, Hsp90s and other chaperone components including p23, complex with ERα to repress its transcriptional activity while maintaining the receptor in a conformation capable of high afffinity ligand binding (14). Additionally, Hsp90s cooperate with the Hsp70 chaperone machinery (13), and therefore a concerted lack of induction of both the Hsp70 and Hsp90 chaperone systems may have a more than additive detrimental affect on accumulation and aggregation of unfolded proteins.

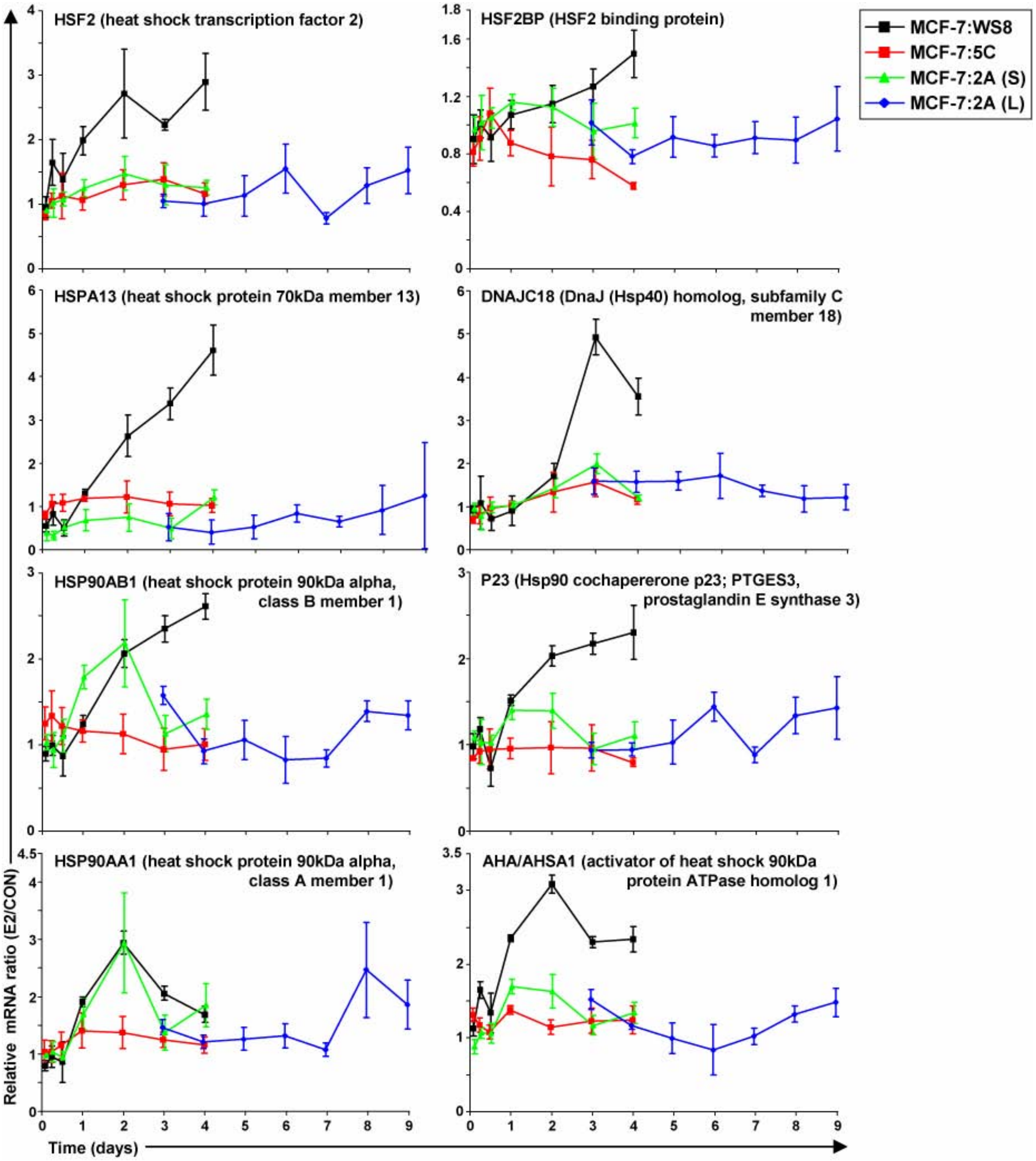
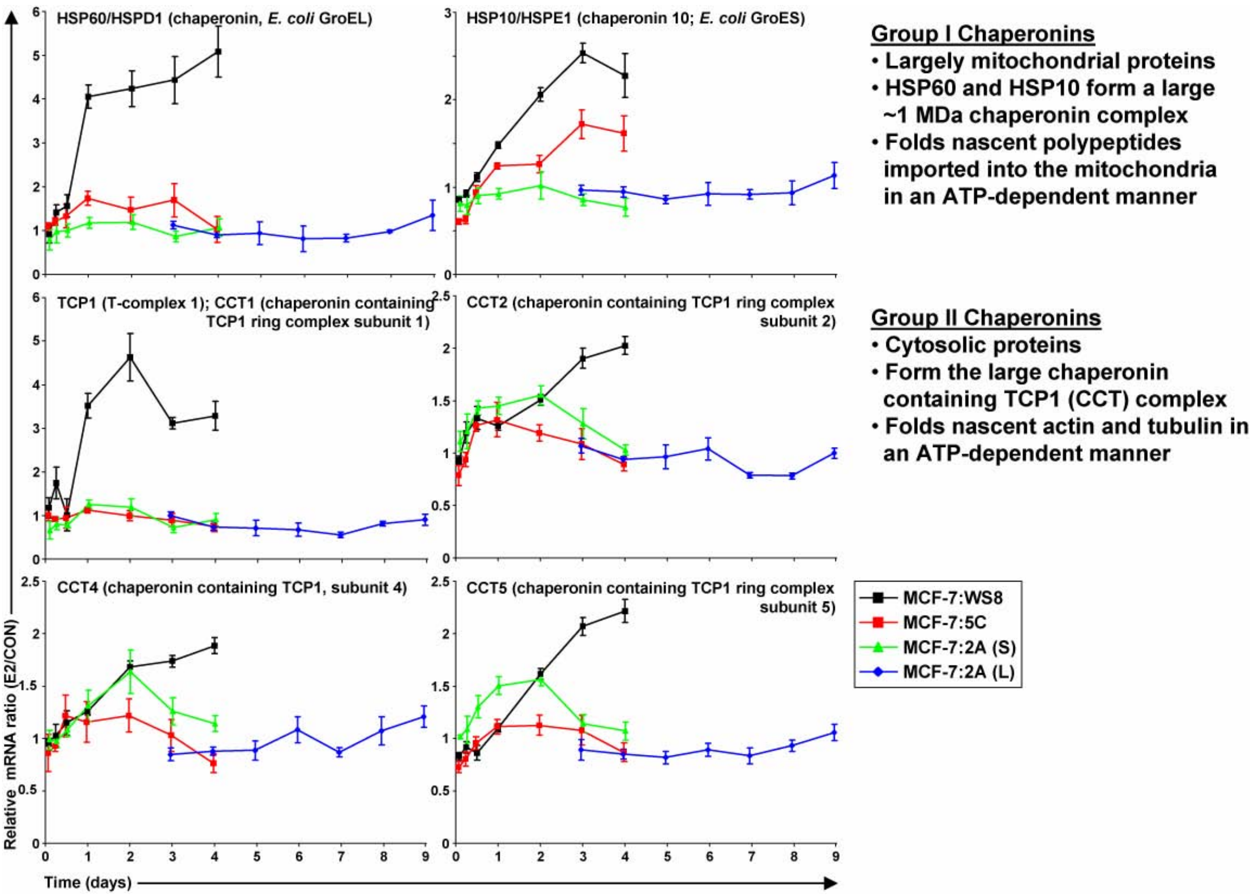


Figure 7. Decreased Induction of Heat Shock Chaperones and Cochaperones.

Further indication of a generalized deficiency in protein folding was illustrated by decreased expression of chaperonins in resistant cells (Fig. 8). Chaperonins form large megadalton two-ring complexes in the shape of a barrel (15). Nascent, non-native polypeptides enter the cavity of the two-ring complex, fold into their native, functional state, and then exit the complex in an ATP-dependent manner. Sometimes, more than one round of folding is required. Chaperonins fall into two classes. Group I chaperonins are mainly localized to the mitochondria and include 2 members, HSP60 (chaperonin, GroEL in *E. coli*), which forms the two-ring barrel, and HSP10 (chaperonin 10, GroES in *E. coli*), which covers the HSP60 two-ring structure like a lid to encapsulate the unfolded protein substrate. Group II chaperonins are localized to the cytosol, include 8 members, and form the chaperonin containing TCP1 (CCT) complex, also known as the TCP1 ring complex (TRiC). The CCT complex does not utilize an HSP10-like cofactor, as this function is already provided by a “built-in” lid that closes in an ATP-dependent manner to encapsulate substrates. The CCT complex folds the cytoskeletal proteins actin and tubulin. In wild-type WS8 cells, E_2-induced the group I chaperonins HSP60 by 5-fold and HSP10 by 2.5 fold, but in resistant cells, E_2 induction of HSP60 was almost completely lacking, as well as for HSP10 in 2A cells, although an intermediary response was observed for HSP10 in 5C cells (Fig. 8). This decreased expression of group I chaperonins would predict a deficiency in folding mitochondrial proteins. Similarly, the group I chaperonins TCP1 (or CCT1), CCT2, CCT4 and CCT5 were selectively E_2-induced in WS8 but not in resistant cells (Fig. 8), predicting accumulation of unfolded cytoskeletal proteins in resistant cells and thus cellular mophology alterations.



Group I Chaperonins
- Largely mitochondrial proteins
- HSP60 and HSP10 form a large ~1 MDa chaperonin complex
- Folds nascent polypeptides imported into the mitochondria in an ATP-dependent manner

Group II Chaperonins
- Cytosolic proteins
- Form the large chaperonin containing TCP1 (CCT) complex
- Folds nascent actin and tubulin in an ATP-dependent manner

Figure 8. Decreased Induction of Chaperonins.

Furthering the theme of resistant cells displaying a generalized deficiency in protein folding, 5C and 2A cells lacked E_2-mediated induction of NPM1 (nucleophosmin, also B23), and MPP11 (also DNAJC2 and yeast Zuo) (Fig. 9). NPM1 is a nucleolar phospho-protein that acts as a molecular chaperone and shuttles between the nucleus and the cytoplasm to participate in ribosomal protein assembly and transport (16). MPP11, a member of the M-phase phosphoprotein family, associates with ribosomes and heterodimerizes with Hsc70 to act as a chaperone of nascent polypeptides as they exit the ribosome (17).

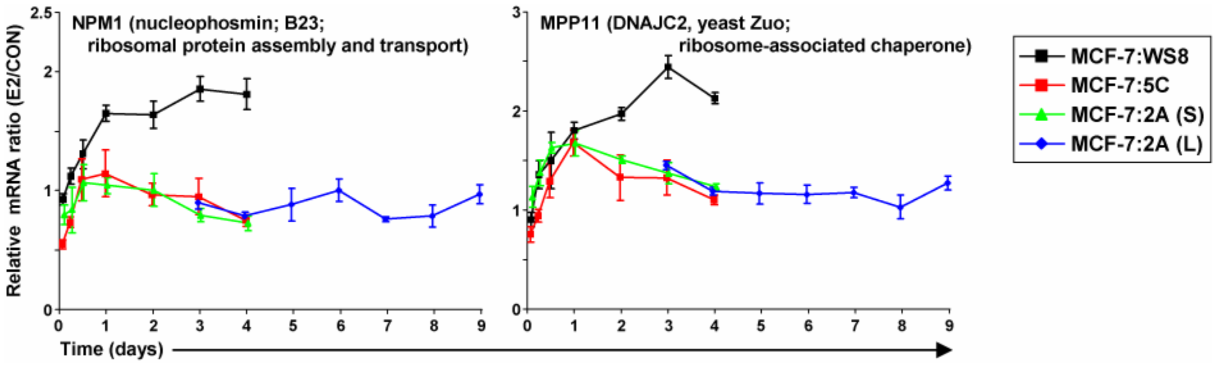


Figure 9. Decreased Induction of Ribosome-associated Chaperones.

Caspase 4

In light of the compelling expression profiles of many genes involved in endoplasmic reticulum stress and unfolded protein stress, and the association between CASP4 and endoplasmic reticulum stress, the functional necessity of CASP4 in mediating apoptosis was investigated.

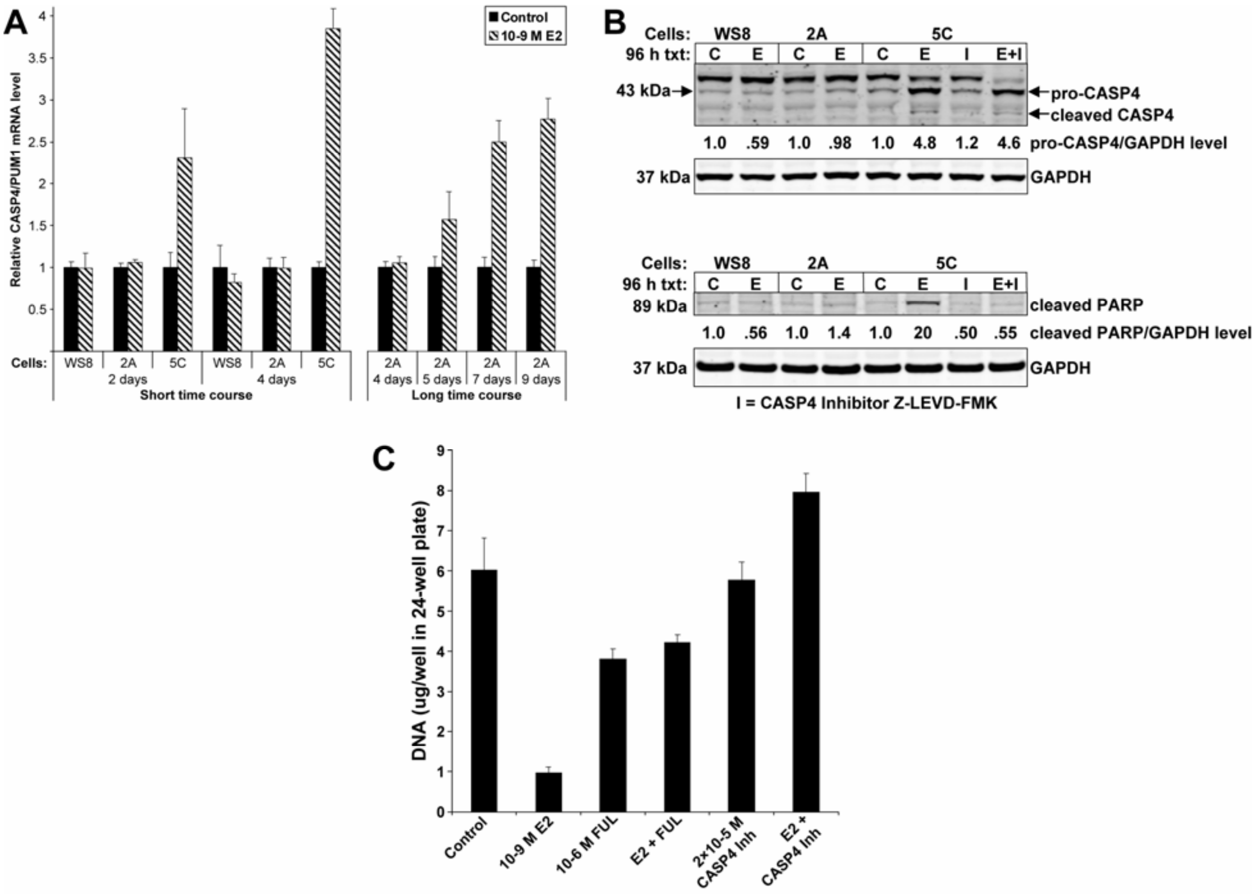


Figure 10. Functional validation of the requirement of CASP4 in E_2-induced apoptosis of 5C cells. (A) E_2-induced CASP4 mRNA by real-time PCR. **(B)** E_2-induced CASP4 protein levels and cleavage in 5C cells at 4 days of E_2 treatment. Also, inhibition of CASP4 blocked E_2-induced PARP cleavage in 5C cells. **(C)** CASP4 blockade reversed E_2-inhibited growth in 5C cells.

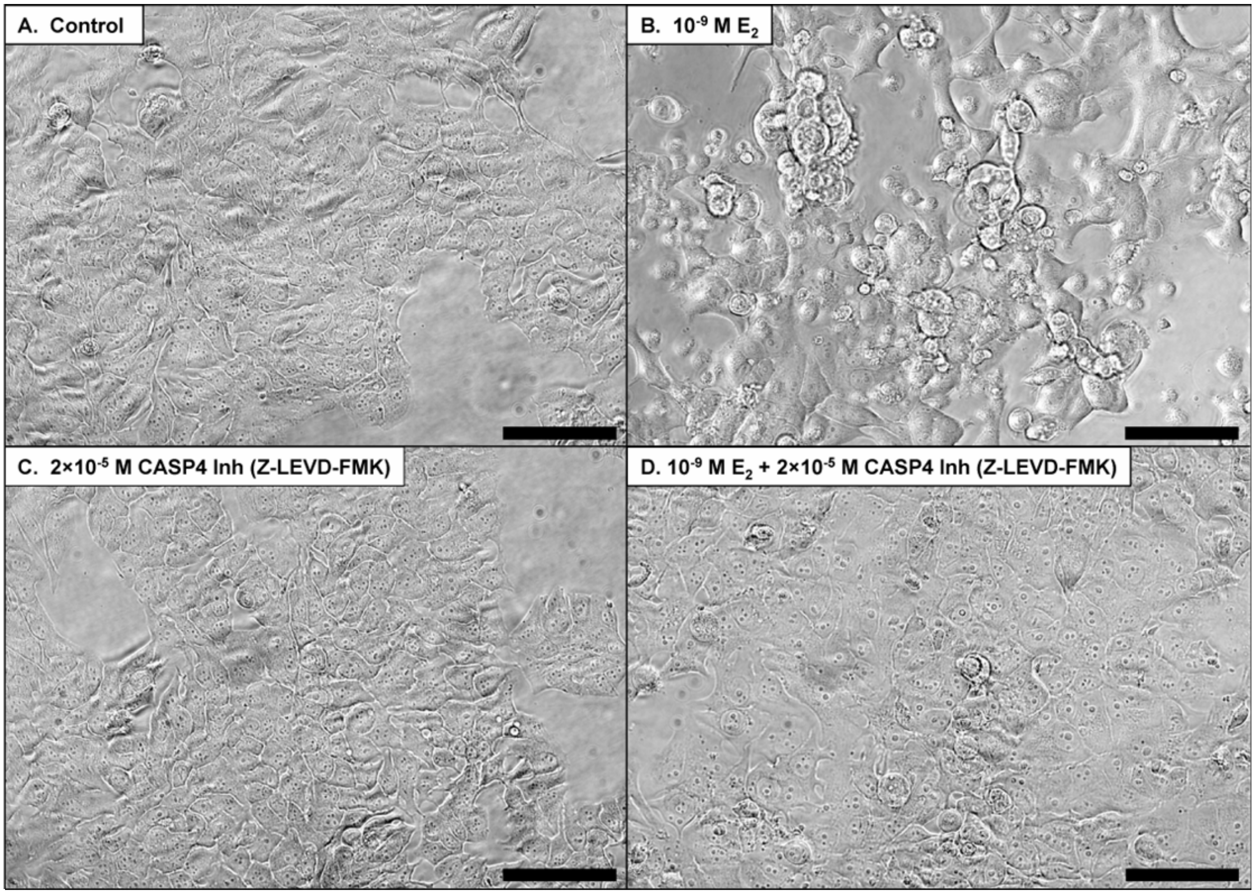

A. Control
B. 10^{-9} M E_2
C. 2×10^{-5} M CASP4 Inh (Z-LEVD-FMK)
D. 10^{-9} M E_2 + 2×10^{-5} M CASP4 Inh (Z-LEVD-FMK)

Figure 11. CASP4 Inhibition Blocked E_2-induced Morphologic Changes Indicative of Apoptosis.

Taken together, E_2-treated MCF-7:5C and MCF-7:2A cells exhibit gene expression profiles of endoplasmic reticulum stress, likely due to accumulation of unfolded proteins in the endoplasmic reticulum, but also show profiles of an overall insufficient ability to fold proteins. This inability to recover from E_2-induced unfolded protein stress likely led to induction of CASP4 in estrogen deprivation-resistant 5C and 2A cells. Since in 5C cells, CASP4 inhibition blocked PARP cleavage, reversed E_2-inhibited growth, and prevented E_2-induced apoptotic morphologic alterations, CASP4 plays a vital role in mediating E_2-induced apoptosis.

KEY FINDINGS

- E_2 induced CASP4, CASP5, CASP10, CASP1, and CASP8 in resistant 5C cells. CASP4 and CASP5 were the first of the caspases to be induced, and these were the only caspases induced in resistant 2A cells (Fig. 3).

- Gene ontology analysis showed deregulated stress response factors were over-represented in the resistant cells. (Fig. 4).

- Examination of stress response gene expression profiles showed a general pattern of induction by E_2 selectively in wild-type MCF-7:WS8 cells, and significantly decreased induction in estrogen-deprivation resistant MCF-7:5C and MCF-7:2A cells (Figs. 5-9).

- The stress response genes indicated that E_2 led to endoplasmic reticulum stress (Fig. 5) and induction of pro-apoptotic Bim (Fig. 6).

- Not only was endoplamic reticulum stress indicated by the expression profiles, but also an overall deficiency in protein folding (Figs. 7-9).

- The endoplasmic reticulum stress-induced CASP4 was functionally required in E_2-induced apoptosis.

  - E_2-induced CASP4 mRNA (Fig. 10A) and protein levels in 5C cells (Fig. 10B).
  - CASP4 was cleaved in E_2-treated 5C cells (Fig. 10B)
  - Inhibition of CASP4 using Z-LEVD-FMK completely blocked E_2-induced PARP cleavage (Fig. 10B), reversed E_2-inhibited growth (Fig. 10C), and prevented E_2-induced apoptotic morphologic alterations in 5C cells (Fig. 11).

These studies will be submitted for publication.

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TASK 2: (FCCC/Jordan) - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2b-3: (Fan and Jordan) – To confirm and validate developing pathways of E_2-induced breast cancer cell survival and apoptosis.

Task 2b is organized into sub-sections 1-4 according to projects led by senior investigators in Dr. Jordan's laboratory that involve deciphering pathways of E_2-induced breast cancer cell survival and apoptosis.

Here we report work completed on Task 2b-3 at the Fox Chase Cancer Center (FCCC) site during year 3 of this COE.

Studies carried out at FCCC by Ping Fan PhD, in the laboratory of Dr. Jordan.

ROLE OF C-SRC TYROSINE KINASE ACTIVITY IN REGULATING APOPTOSIS INDUCED BY E_2 IN LONG-TERM ESTROGEN DEPRIVATION-RESISTANT BREAST CANCER CELLS

Introduction

Recent evidence has established the non-receptor tyrosine kinase, c-Src, as a critical component of multiple signaling pathways that regulate proliferation, survival, angiogenesis and metastasis (1). Increased Src activity is believed to play an important role in development and progression in breast cancer. Furthermore, elevated expression of Src has been associated with poor prognosis (2). In tamoxifen-resistant MCF-7 cells, c-Src tyrosine kinase activity was increased and promoted cellular invasion and motility relative to tamoxifen-sensitive cells (3,4). Further, c-Src was shown to be functionally critical in mediating tamoxifen resistance since blocking its activity has been shown to reverse tamoxifen resistance (5). c-Src activation by steroid hormone receptors, ERα, AR and PR, have all been reported through different mechanisms (6-8). Our previous results showed that physiological concentrations of E_2 induce apoptosis in MCF-7:5C breast cancer cells (9). To synergize with E_2 and investigate the function of c-Src tyrosine kinase in apoptosis induced by $E_{2,}$ we treated antihormone-resistant cells with E_2 plus the c-Src tyrosine kinase inhibitor PP2.

WORK ACCOMPLISHED

Growth effects of the c-Src tyrosine kinase inhibitor PP2 in antihormone resistant cells

We studied the concentration response of the c-Src tyrosine kinase inhibitor PP2 in estrogen-responsive MCF-7:WS8, and in estrogen deprivation-resistant MCF-7:2A and MCF-7:5C cells. MCF-7:5C cells were more sensitive to PP2 than MCF-7:WS8 and MCF-7:2A cells (Fig. 1A). Examination of the c-Src phosphorylation levels showed that c-Src was highly phosphorylated at Tyr-416 in MCF-7:5C cells compared to MCF-7:WS8 cells (Fig. 1B). Since MCF-7:5C cells displayed the most sensitivity to PP2, and E_2 inhibits growth through inducing apoptosis in these cells, the effect of combining PP2 with E_2 on growth of MCF-7:5C cells was examined. Interestingly, while E_2 (10^{-9} M) alone dramatically inhibited growth, and PP2 alone modestly inhibited growth, the addition of PP2 to E_2 did not further block growth compared to E_2 alone (Fig. 1C). Surprisingly, the MCF-7:5C cells appeared healthy when cultured in E_2 plus PP2 over the long-term, and selection under these conditions for 2 months produced a polyclonal cell line that grew faster compared to long-term E_2 alone- or PP2 alone-selected MCF-7:5C polyclonal cell lines. The implication of these results are potentially clinically important; that a Src inhibitor should not be combined with E_2 in the treatment of advanced aromatase inhibitor-resistant breast cancer.

Figure 1.

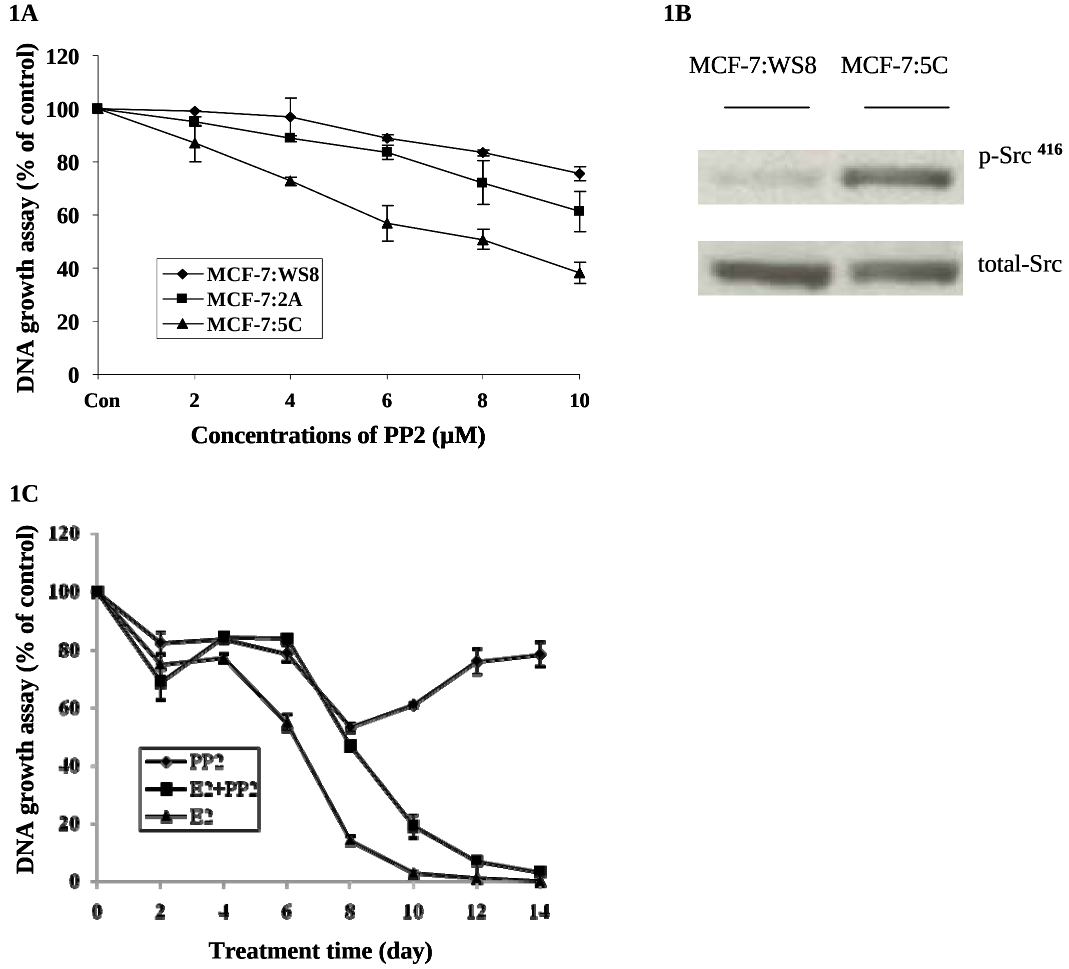

Figure 1. (A) Responses of estrogen-responsive MCF-7:WS8 cells and of estrogen deprivation-resistant MCF-7:2A and MCF-7:5C cells to the c-Src inhibitor PP2**.** MCF-7:WS8, MCF-7:2A and MCF-7:5C cells were treated with different concentrations of the c-Src inhibitor PP2 for 7 days in phenol red-free RPMI 1640 containing 10% charcoal-stripped serum. Cells were harvested and total DNA was determined using the DNA-binding fluorescent dye Hoechst 33528 and comparison to a standard curve of serially-diluted calf thymus DNA. Data shown are representative of at least three separate experiments with similar results. *P* = 0.336, MCF-7:2A compared with MCF-7:WS8. *P* = 0.048, MCF-7:5C compared with MCF-7:WS8. **(B)** Lysates of MCF-7:WS8 and MCF-7:5C cells were harvested**.** The antibody to phospho-c-Src $^{\mathrm{Tyr416}}$ was used to examine the phosphorylation level of c-Src. Total c-Src was used as loading control. **(C)** Time courses of MCF-7:5C cells treated with PP2, E_2, and E_2 plus PP2. MCF-7:5C cells were seeded in the 6-well plates with 10,000 cells/well in triplicate. The next day, cells were treated with PP2 (5 μM), E_2 (1 nM), and E_2 (1 nM) plus PP2 (5 μM) in phenol red-free RPMI 1640 containing 10% charcoal-stripped serum. Cells were harvested at the indicated time points and total DNA was determined as in (*A*). Data shown are representative of at least three separate experiments with similar results.

ERα expression changes in response to c-Src inhibition

c-Src is an important adapter protein that interacts with estrogen receptor alpha (ERα). We detected changes in ERα expression in MCF-7:5C cells after short-term treatment and long-term selection with E_2, PP2, and E_2 plus PP2 producing polyclonal cell lines. It was interesting to find that blocking c-Src tyrosine kinase activity increased ERα protein levels after 24 h treatment (Fig. 2A). This higher expression level of ERα was maintained over the long-term (more than several months) (Fig. 2B). The mechanism by which blocking Src tyrosine kinase up-regulated ERα is unknown. E_2 and E_2 plus PP2-treated cells down-regulated ERα levels to such a low level that it was not visualized in these lanes (data not shown). However, longer exposure times did reveal an ERα band in these lanes. The very low levels of ERα were expected due to E_2-mediated down-regulation. Hence, PP2 was not capable of overcoming this E_2-mediated ERα down-regulation (Fig. 2A -2C).

Figure 2

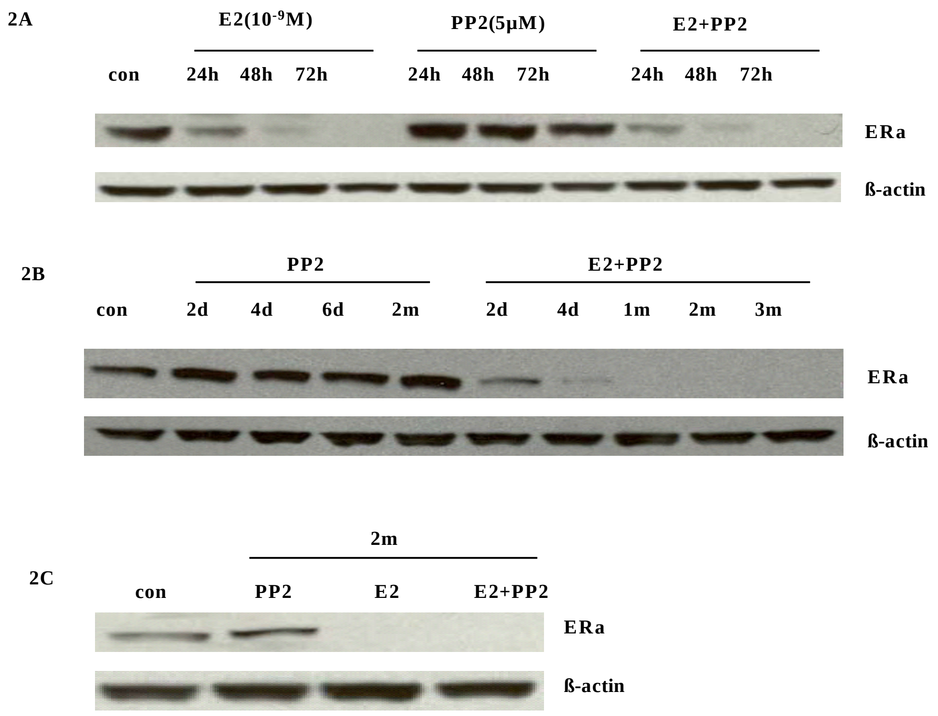

Figure 2. Cells were harvested after the indicated treatment times. Immunoblots probed with ERα antibody were used to detect the changes in ERα expression. β-actin was used as a loading control.

E_2 promoted growth of long-term E_2 plus PP2-selected antihormone-resistant cells

Since the c-Src inhibitor plus E_2 changed the growth characteristics of MCF-7:5C cells, their response to E_2 alone needed to be re-examined. To our surprise, long-term selection of MCF-7:5C cells with PP2 plus E_2 produced a polyclonal cell line that was not only resistant to E_2 -induced apoptosis, but instead was dramatically growth stimulated by E_2 (Fig. 3A). This growth stimulation by E_2 was blocked by the pure antiestrogen ICI 182,780, confirming that ER yet mediated proliferation despite its low expression (Fig. 3B). Thus, ERα must still be expressed in these cells, but at very low levels. E_2 yet induced apoptosis in long-term PP2-treated MCF-7:5C cells, implying that E_2's apoptotic trigger may not require activated c-Src. In E_2 alone-treated cells, E_2 both induced apoptosis and stimulated growth, but these effects cancelled each other out in terms of total DNA content per well (Fig. 3A). To assess whether long-term E_2 plus PP2 treatment could change the estrogen responsiveness of other estrogen deprivation-resistant cells, MCF-7:2A cells were selected long-term under these conditions. E_2 stimulation of MCF-7:2A cells selected long-term with E_2 plus PP2 showed a similar response as did the

MCF-7:5C cells selected under the same condition, E_2 caused modest growth instead of delayed apoptosis (Fig. 3C).

Figure 3

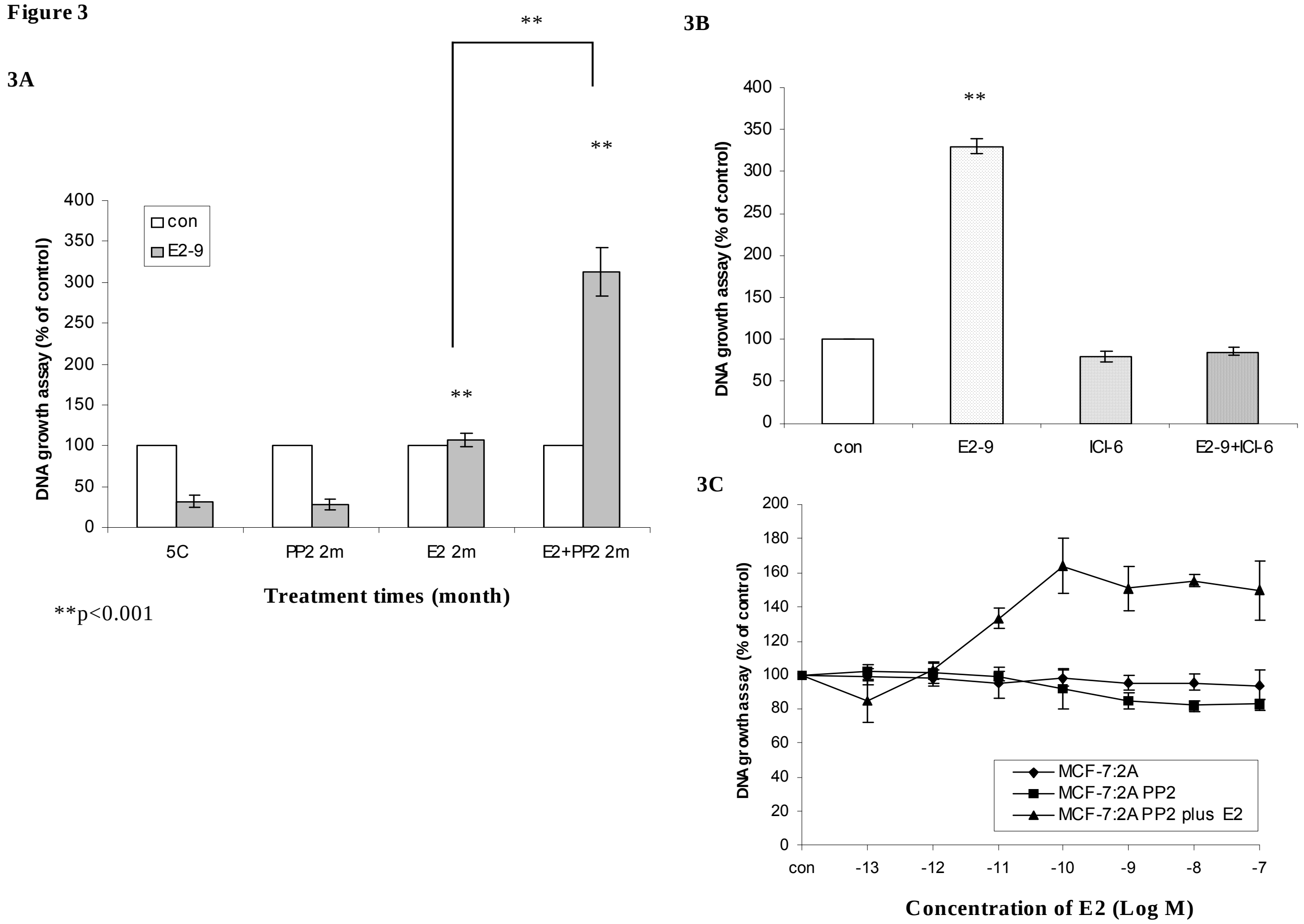

Figure 3. (A) MCF-7:5C cells, and MCF-7:5C cells selected long-term (2 months) with PP2, E_2, and E_2 plus PP2 cells producing polyclonal cell lines, were seeded in 24-well plates at 15,000 cells/well in triplicate. The next day, cells were treated with E_2 (1 nM) in phenol red-free RPMI 1640 medium containing 10% charcoal-stripped serum without any other compounds in the medium. The cells were harvested after 7 days treatment and total DNA was determined using the DNA-binding fluorescent dye Hoechst 33528 and comparison to a standard curve of serially-diluted calf thymus DNA. $P < 0.001$ ** compared with MCF-7:5C cells or as indicated. **(B)** E_2 plus PP2-selected MCF-7:5C were seeded in 24-well plates at 15,000 cells/well in triplicate. The following day, cells were treated with E_2 (1 nM), ICI 182,780 (10^{-6} M), and E_2 (1 nM) plus ICI 182,780 (10^{-6} M). The same method was used to measure DNA mass as in (*A*). $P < 0.001$ ** compared with control. **(C)** MCF-7:2A cells, PP2-selected MCF-7:2A cells, and PP2 plus E_2-selected MCF-7:2A cells were seeded in 24-well plates at 15,000 cells/well in triplicate. The next day, cells were treated with E_2 (1 nM) in phenol red-free RPMI 1640 medium containing 10% charcoal-stripped serum without any other compounds in the medium. The cells were harvested after 7 days of treatment and total DNA was determined as in (*A*). Data shown are representative of at least three separate experiments with similar results. $P < 0.01$, PP2 plus E_2-selected cells compared to unselected MCF-7:2A cells. Data shown are representative of at least three separate experiments with similar results.

SERMs promoted growth of long-term E_2 plus PP2-selected MCF-7:5C cells

Since E_2 stimulated instead of inhibited growth of E_2 plus PP2-selected MCF-7:5C cells, the growth responses of these cells to the complete antiestrogen ICI 182,780 and the selective estrogen receptor modulators (SERMs) 4-hydroxytamoxifen (4-OHT), endoxifen, EM652, and raloxifene. ICI 182,780 did not significantly affect growth of the E_2 plus PP2-selected MCF-7:5C cells, but all of the SERMs exhibited dramatically increased agonist activity by stimulating proliferation in these cells (Fig. 4A). This proliferative effect could be blocked by ICI 182,780, which implied that the SERMs enhanced growth through the ER (Fig. 4B). It is under investigating whether estradiol and SERMs used the similar or different growth pathways to stimulate cells growth.

Figure 4

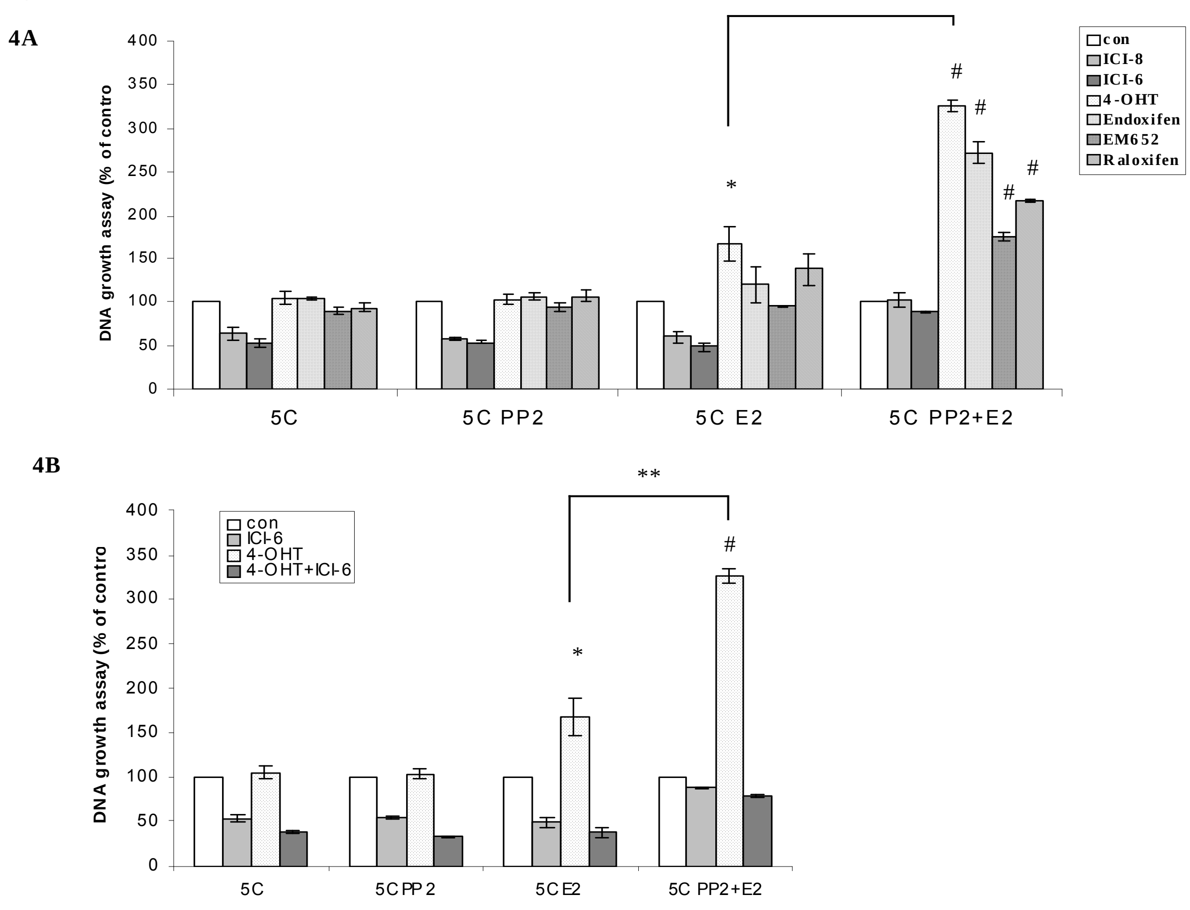

Figure 4. MCF-7:5C cells and PP2-, E_2-, and E_2 plus PP2-selected MCF-7:5C cells were seeded in 24-well plates at 15,000 cells/well in triplicate. The following day, cells were treated as indicated with ICI 182,780 (10^{-8} M and 10^{-6} M) and the SERMs 4-OHT, endoxifen, EM652, and raloxifene (all at 10^{-6} M) in phenol red-free RPMI 1640 medium containing 10% charcoal-stripped serum without any other compounds in the medium. The cells were harvested after 7 days of treatment and total DNA was determined using the DNA-binding fluorescent dye Hoechst 33528 and comparison to a standard curve of serially-diluted calf thymus DNA. Data shown are representative of at least three separate experiments with similar results. $P < 0.01$, * compared with MCF-7:5C cells, $P < 0.001$ ** compared with MCF-7:5C cells or as indicated, $P < 0.0001$ # compared with MCF-7:5C cells (*A* and *B*).

KEY FINDINGS

- Estrogen deprivation-resistant MCF-7:5C cells exhibited high levels of activated c-Src, leading to greater sensitivity to the c-Src inhibitor PP2 compared to wild-type MCF-7:WS8 cells. This demonstrates that the c-Src tyrosine kinase was involved in adaptation of MCF-7 cells to estrogen deprivation (Fig. 1).

- Quite surprisingly, selection of MCF-7:5C cells under E_2 plus PP2 conditions produced a cell line in which E_2 did not induce apoptosis, and instead dramatically stimulated growth (Fig. 3).

- Selection of MCF-7:5C cells under E_2 plus PP2 conditions also allowed SERMs to manifest greater agonist activity by promoting growth (Fig. 4).

Conclusions and Future Directions

In summary, contrary to our original hypothesis that the c-Src inhibitor PP2 could act with E_2 to additively or synergistically block growth of advanced estrogen deprivation-resistant MCF-7:5C breast cancer cells growth, long-term treatment with PP2 plus E_2 gradually reversed E_2's apoptotic effects, and actually allowed E_2 to stimulate growth, although this selective pressure also resulted in very low ERα levels. Additionally, long-term treatment of MCF-7:5C with PP2 plus E_2 selected for cells that exhibited greater agonist activity of SERMs in terms of growth stimulation. These data raise a concern regarding the ubiquitous use of c-Src inhibitors in all forms of antihormone-resistant breast cancer. Soon, c-Src inhibitors will be moved into clinical trials. However, if we validate that c-Src inhibitors reverse E_2-induced apoptosis in 3D cell culture and xenograft model experiments, these results may have important clinical implications for appropriately utilizing c-Src inhibitors in Phase II (advanced) antihormone-resistant breast cancers.

We will investigate mechanisms underlying c-Src inhibition in converting responses to E_2 from apoptosis to growth. Our data indicated that this conversion was not simply the result of blocking c-Src tyrosine kinase activity since c-Src was not phosphorylated at Ser-416 in either PP2- or E_2 plus PP2-selected MCF-7:5C cells (data not shown). Therefore, the combination of E_2 plus c-Src inhibition must target other critical factors to reverse E_2 responses from apoptosis to growth.

As in Task 4, we will use Agilent microarrays to analyze genome-wide changes in gene expression in E_2 plus PP2-selected MCF-7:5C cells compared to unselected MCF-7:5C cells treated plus/minus E_2. This will allow identification of a gene signature and biomarkers that could be used to decipher which patient's disease could effectively be treated with c-Src inhibitors. Furthermore, we will likewise determine global changes in gene expression in these cells in response to the active tamoxifen metabolite 4-OHT. Finally, we will functionally validate the involvement of the identified differentially expressed genes in mediating growth responses of the E_2 plus PP2-selected MCF-7:5C cells.

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TASK 2. FCCC/Jordan - To elucidate the molecular mechanism of E_2 induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.

Task 2b-4: (Sengupta and Jordan) – To confirm and validate developing pathways of E_2-induced breast cancer cell survival and apoptosis.

Task 2b is organized into sub-sections 1-4 according to projects led by senior investigators in Dr. Jordan's laboratory that involve deciphering pathways of E_2-induced breast cancer cell survival and apoptosis.

Here we report work completed on Task 2b-4 at the Fox Chase Cancer Center (FCCC) site during year 3 of this COE.

Studies carried out at FCCC by Surojeet Sengupta PhD, in the laboratory of Dr. Jordan.

ROLE OF XBP1 (X-BOX BINDING PROTEIN-1) IN MODULATING ESTROGEN-MEDIATED GROWTH OF BREAST AND ENDOMETRIAL CANCER CELLS BY REGULATING BCL2 (B CELL LYMPHOMA-2) AND ITS MODE OF ACTION

Prior Progress

XBP1 (X-box binding protein 1), is a known estrogen regulated gene which is highly co-expressed with ERα (estrogen receptor alpha) in breast cancer patients. Previously (in the Year 2 report), and again shown here for convenience, we showed that depletion of XBP1 can markedly inhibit 17β-estradiol (E_2) -induced growth of ERα-positive breast (Fig. 1B) and endometrial cancer cells and also down-regulates the expression levels of Bcl-2 (B Cell Lymphoma-2) in breast and endometrial cancer cells (Fig. 1C and D). In addition we showed that ERα as well as XBP1 is recruited to the putative XBP1-binding element in the promoter region of the Bcl-2 gene (Year 2 report).

WORK ACCOMPLISHED

Effect of Bcl-2 depletion on E_2-stimulated growth of MCF-7 and ECC1 cells

To confirm that the growth inhibitory effects of XBP1 depletion were actually mediated by Bcl-2 levels, we evaluated the direct effect of Bcl-2 depletion on the growth of MCF-7 and ECC1 cells in presence or absence of E_2. We used a pool of four small interfering (si) RNAs to knock-down Bcl-2 expression in MCF-7 and ECC1 cells, and investigated its effect in E_2-induced growth. The cells transfected with Bcl-2 siRNA or control siRNA were re-seeded in 24 well plates and growth of the cells was monitored over a four day period in the presence or absence of 1 nM E_2. E_2-induced growth of MCF-7 and ECC1 cells was inhibited drastically in the Bcl-2 –depleted cells as compared to growth of control siRNA-transfected cells (Fig. 2A and 2B). These data suggested that Bcl-2 depletion plays a critical role in mediating E_2-induced growth in ERα-positive breast and endometrial cancer cells.

Effect of XBP1 over-expression on Bcl-2 expression in MCF-7 cells

To further confirm the direct effects of XBP1 on the regulation of Bcl-2 expression, we transiently over-expressed XBP1 in MCF-7 cells and monitored the protein expression of Bcl-2. Our data (Fig. 3) showed that over-expression of XBP1 led to moderately higher levels of Bcl-2 expression compared to cells over-expressing an unrelated protein Lac-Z. However, the increase in Bcl-2 level was evident only in the cells treated with E_2, which indicated that there are other E_2-regulated factors needed to up-regulate Bcl-2 expression.

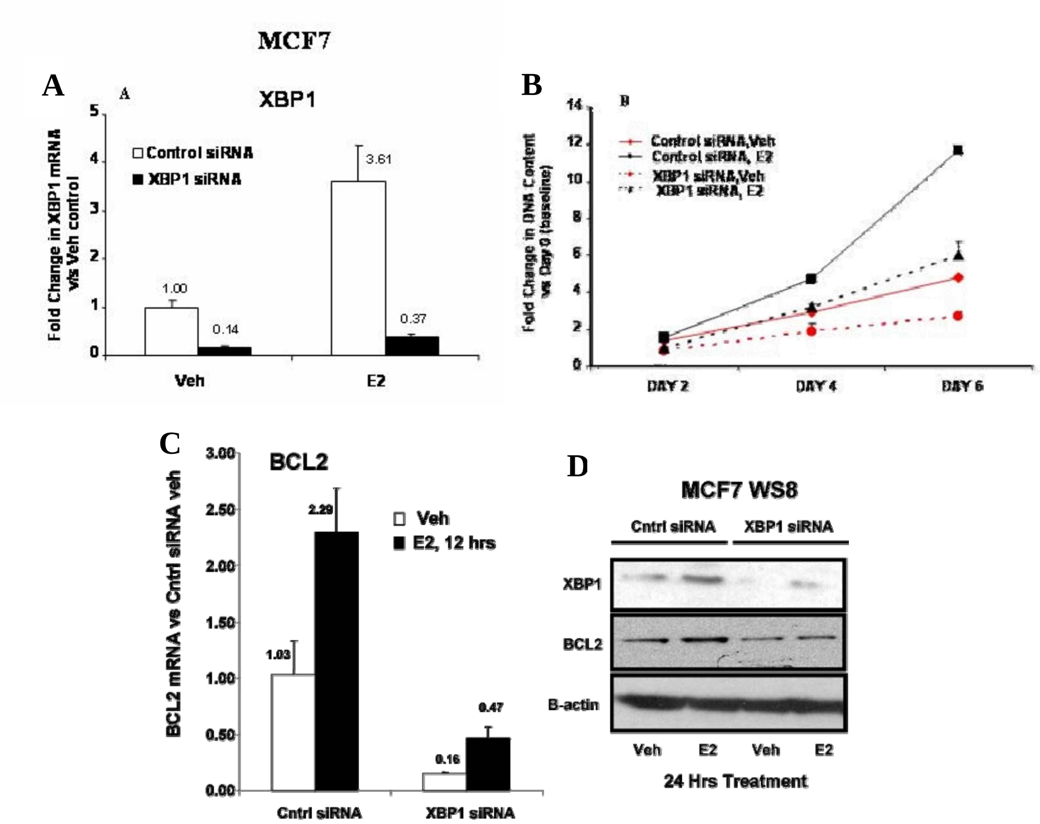

Figure 1. **(A)** MCF-7 cells, transfected with XBP1 siRNA or control siRNA, were treated with vehicle or E_2 (1 nM) for 24 hrs and the extent of knockdown was assessed using quantitative real-time PCR compared with vehicle-treated control siRNA cells **(B)**. Subsequently, cells were re-seeded and the growth of the cells was monitored over a six day period. Total DNA content was measured as a marker of growth and the fold change in DNA content was calculated compared to the number of cells at the time of start of the treatment (baseline) **(C)**. MCF-7 cells were transfected with XBP1 siRNA or control siRNA. Bcl-2 mRNA expression was measured using quantitative real-time PCR after 12 hrs, and **(D)** Bcl-2 protein levels were measured by Western blotting after 24 hrs of E_2 treatment.

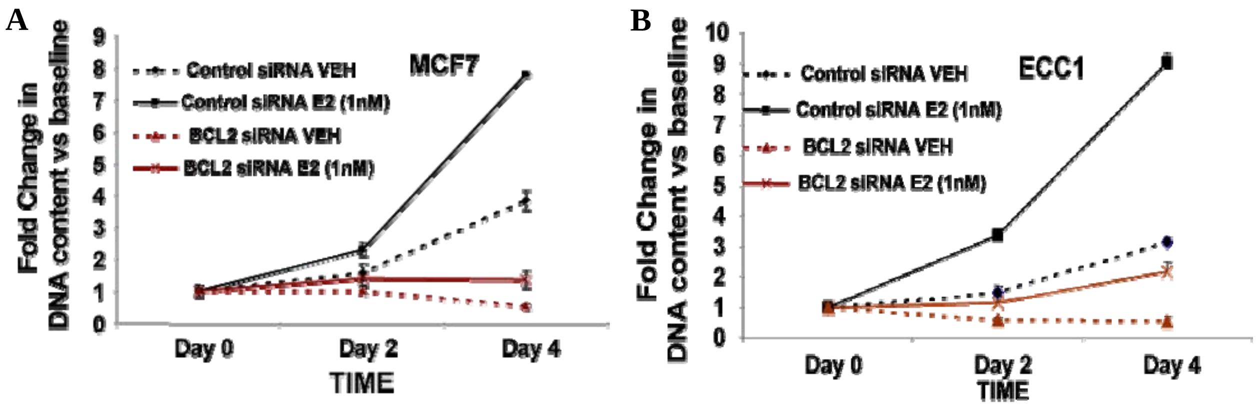

Figure 2. **(A)** MCF-7 cells **(B)** and ECC1 cells were transfected with Bcl-2 siRNA or control siRNA, and subsequently, cells were re-seeded and the growth of the cells was monitored over a four day period. Total DNA content was measured as a marker of growth and the fold change in DNA content was calculated compared to the number of cells at the time of start of the treatment (baseline).

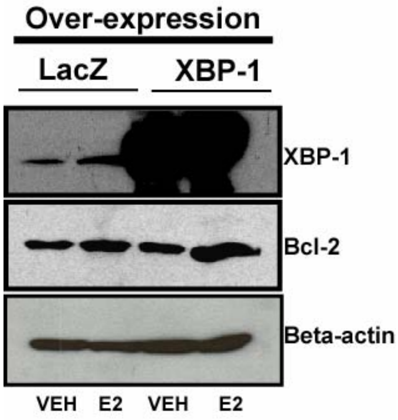

Figure 3. XBP1 or Lac-Z expressing plasmid was transiently over-expressed in MCF-7 cells. The cells were then treated with either 0.1% ethanol (Veh) or 1 nM E_2 for 24 hrs. The protein lysates were collected in RIPA buffer in the presence of protease and phosphatase inhibitors. Western blot was performed using standard methods.

Interaction of XBP1 with ER alpha in MCF-7 breast cancer cells

Next, we evaluated the possibility of a physical interaction between XBP1 and ERα in MCF-7 cells in the absence or presence of E_2. MCF-7 cells were grown for three days in phenol red-free media containing 10% charcoal-stripped media before treating them for vehicle or E_2 for another 24 hrs. Immunoprecipitation was performed using antibodies against XBP1 followed by Western blotting for ERα. We observed (Fig. 4) that physical interaction between XBP1 and ERα was evident in absence as well as in the presence of E_2. It is pertinent to mention here that previously we also have observed recruitment of XBP1 and ERα to the BCL2 promoter region in the presence or absence of 1 nM E_2.

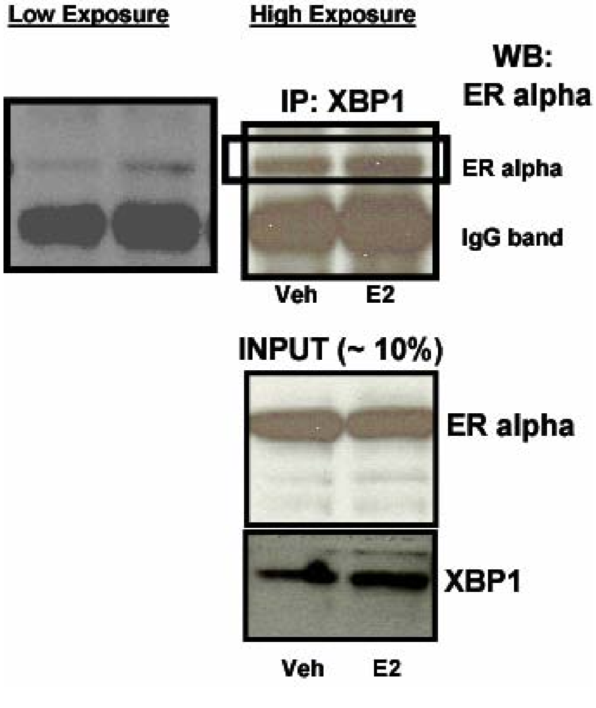

Figure 4. MCF-7 cells were treated with either vehicle (Veh) or 1 nM E_2 for 24 hrs. Protein lysates were prepared in RIPA buffer containing protease and phosphatase inhibitors. Protein lysates (250 µg) were pre-cleared with normal rabbit IgG and protein G plus agarose beads. The lysates were immunoprecipitated with XBP1 antibody (2 µg; Santa Cruz, cat# sc-7160). The immunoprecipitated proteins were probed using a monoclonal antibody against ERα (Neomarkers, Ab-15).

XBP1 is not recruited at the Estrogen Response Element (ERE) region of the PS2 promoter in MCF-7 breast cancer cells

Following the observation that XBP1 and ERα proteins physically interacted, we evaluated the possibility of XBP1 recruitment to EREs, which are the DNA sequences to which ERα directly binds. To test this hypothesis we performed the ChIP assay using XBP1 and ERα antibodies at the PS2 gene promoter, which is a well known to contain an ERE. Recruitment of XBP1 was not observed (Fig. 5A) at the PS2 promoter ERE region. However, as expected ERα was strongly recruited in an E_2-dependent manner to this region of the PS2 promoter (Fig. 5A).

Effect of depletion or over-expression of XBP1 on ERE-luciferase reporter assay in MCF-7 breast cancer cells

To further investigate the effect of XBP1 levels on transcriptional activity of ER via a consensus ERE, we performed an ERE-luciferase reporter assay in MCF-7 cells, either transiently over-expressing XBP1 or depleted of XBP1 using siRNA. No differences were observed (Fig 5B and 5C) in transcriptional activity from the ERE-luciferase reporter in the cells either over-expressing XBP1 or the cells depleted of XBP1 as compared to their respective controls. Based on these results, together with the observation that XBP1 was not recruited to the ERE-region of the PS2 promoter, we conclude that XBP1 does not modulate classical transcriptional activity of ERα mediated through direct binding of an ERE. However, we can not exclude the possibility that XBP1 could modulate ERα transcriptional activity via non-classical tethering to other transcriptional response elements such as AP-1 and SP-1.

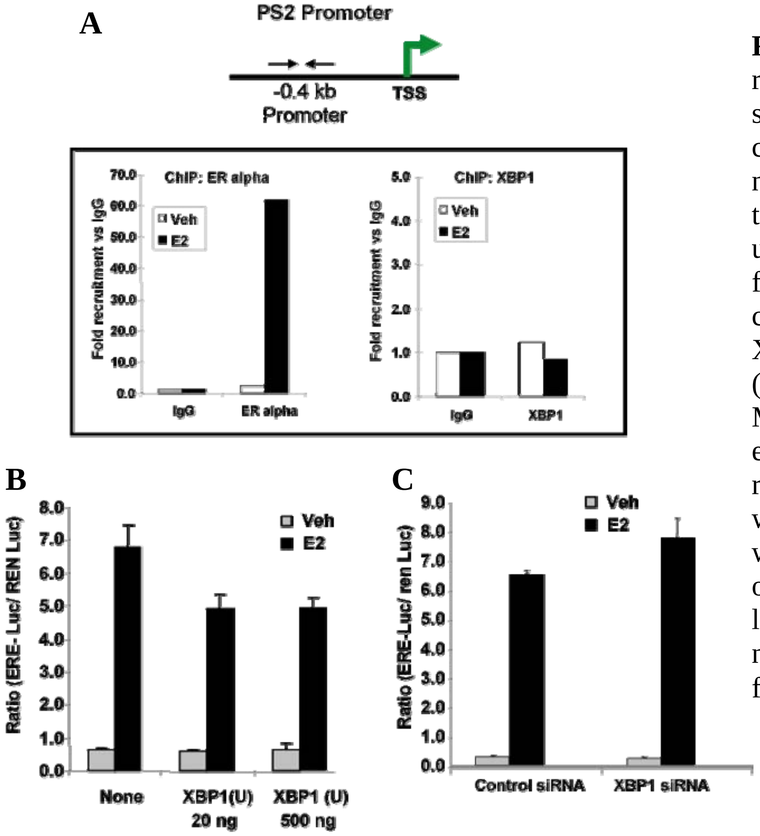

Figure 5. (A) MCF-7 cells were grown in phenol red-free media supplemented with 10% charcoal-stripped bovine serum for three days before the cells were treated with either vehicle (Veh) or 1 nM E_2 for 45 m followed by fixing and collecting the cells for a ChIP assay. Normal rabbit IgG was used as a control. The data are represented as fold-change in recruitment of ERα or XBP1 compared to IgG. Antibodies against ERα and XBP1 were purchased from Santa Cruz Biotech (cat # sc-543 and sc-7160 respectively). **(B)** MCF-7 cells were transfected with an XBP1-expression plasmid and an ERE-luciferase reporter assay was performed. Renilla luciferase was used to normalize for transfection in each well. **(B)** MCF-7 cells were treated with control or XBP1 siRNA before performing an ERE-luciferase assay in the absence or presence of 1 nM E_2. Renilla luciferase was used to normalize for transfection in each well**.**

Effect of stable over-expression of XBP1 on paclitaxel (taxol) induced toxicity in MCF-7 cells

To further understand the role of XBP1 in cell survival, we developed MCF-7 cell clone that stably expressed the unspliced mRNA variant of XBP1 under control of the CMV promoter for constitutive expression. The mRNA expression level of XBP1 in this particular MCF-7 clone (MCF-

7/XBP1/Unspliced) was ~2.5 fold higher than the MCF-7 parental cell line (Fig. 6A) as assessed by quantitative real-time PCR. To study the effect of chemotherapeutic toxicity in these cells, we treated the MCF-7/XBP1/Unspliced cells with different concentrations of paclitaxel for 48 hours and assessed the toxicity in terms of percentage survival of the vehicle treated cells. In parallel, MCF-7 parental cells were treated identically. The MCF-7/XBP1/Unspliced cells were significantly more resistant to paclitaxel induced toxicity compared to the MCF-7 parental cells (Fig. 6B). This indicates that higher levels of XBP1 protected MCF-7 breast cancer cells from paclitaxel induced cell toxicity.

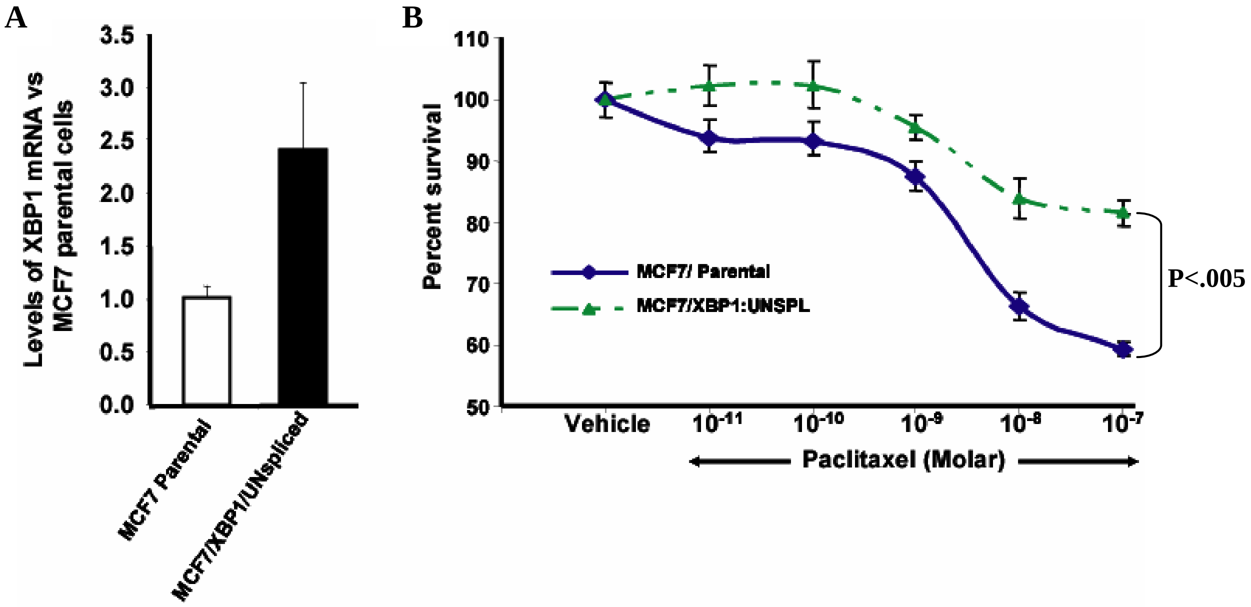

Figure 6. (A) MCF-7 parental and MCF-7/XBP1/Unspliced cells were cultured in phenol red-free RPMI 1640media containing 10% charcoal-stripped bovine serum. Total RNA was isolated and levels of XBP1 mRNA were assessed using quantitative real time PCR. **(B)** MCF-7 parental and MCF-7/XBP1/Unspliced cells were treated with vehicle (0.1 % DMSO) or different concentration of paclitaxel for 48 hrs before assessing the cell numbers using a DNA-based assay. The data are represented in terms of % survival, compared to vehicle-treated cells (100 %).

In summary, this study illustrates that XBP1 is a critical player in the regulation of E_2-stimulated growth and expression of the proto-oncogene Bcl-2 in ERα-positive breast cancer cells. We confirmed that inhibition of growth due to XBP1 depletion was mediated by low levels of Bcl-2. This study also suggested that XBP1 did not modulate the classical transcriptional activity of ERα mediated by direct binding to an ERE. Further studies will be necessary to understand the precise mechanism of action of XBP1. Understanding XBP1's mechanism of action may provide novel drug targets for hormone resistance in ERα-positive breast and endometrial cancers.

KEY FINDINGS

- XBP1 depletion dramatically inhibited E_2-stimulated growth of ERα-positive breast and endometrial cancer cells.

- Depletion of XBP1 down-regulated the expression of Bcl-2 levels in MCF-7 and ECC1 cells.

- Depletion of Bcl-2 levels also dramatically inhibited E_2-stimulated growth of ERα-breast and endometrial cancer cells, suggesting that the growth inhibitory effects of XBP1 depletion may have been mediated at least partly through decreased Bcl-2 levels.

- Over-expression of XBP1 in MCF-7 cells modestly increased Bcl-2 levels selectively in the presence of E_2.

- XBP1 physically interacted with ERα protein in MCF-7 cells in a ligand-independent manner.

- XBP1 levels (depletion or over-expression) do not modulate classical transcriptional activity of ERα mediated through binding of an ERE.

- MCF-7 cells stably transfected with XBP1 are significantly more resistant to paclitaxel (taxol) induced toxicity, suggesting higher expression of XBP1 can potentially reduce the therapeutic efficacy of chemotherapeutic agents.

Future Directions

Future work will focus on understanding the precise mechanisms by which XBP1 may influence the E_2-induced growth of MCF-7 cells and E_2-induced apoptosis in estrogen deprivation-resistant MCF-7:5C and MCF-7:2A cells.

TASK 3: (GU/Riegel and Wellstein) - To decipher cellular signaling pathways using proteomics and to mesh proteomics and mRNA analysis.

Here we report work completed on Task 3 at the Georgetown University (GU) site during year 3 of this COE.

Studies carried at GU in the laboratory of Anna Riegel PhD and the laboratory of Anton Wellstein MD PhD, with support from Benjamin Kagan PhD, and H. Zu PhD.

PROTEOMIC AND BIOINFORMATIC PATHWAY ANALYSIS OF 17B-ESTRADIOL (E_2)-INDUCED GROWTH VERSUS APOPTOSIS IN ESTROGEN-RESPONSIVE MCF-7 AND ESTROGEN DEPRIVATION-RESISTANT MCF-7/5C CELLS

Under this task we aim to identify signaling events that control estrogen-induced apoptosis in contrast to the induction of cell growth. A crucial model for this is a comparison between MCF-7/5C, a long-term estrogen deprivation-resistant variant line of breast cancer MCF-7 cells that undergo apoptosis in response to E_2, whereas wild-type MCF-7 cells will grow in response to E_2. The major goal during the third year of the project at the GU site was to use proteomics data and analysis to identify pathways and candidate proteins that control E_2-induced apoptosis versus growth. Specifically we based our analysis on proteins interacting with the nuclear-receptor co-activator AIB1 (Amplified in Breast Cancer 1) and phospho-tyrosine containing signal complexes early after E_2 treatment. From a comparison of these experimental data sets obtained from MCF-7 versus MCF-7/5C cells ± E_2 at different time points and from a computationally-derived global AIB1-interacting network, we obtained altered pathways that are involved in the differential response to E_2.

We report here on the new data and new analyses generated during year 3. For some of the experimental details we refer the reader to the reports from year 1 and year 2.

WORK ACCOMPLISHED

AIB1-interacting and phopho-tyrosine complex proteins

As proposed in the original experimental plan, we isolated proteins from MCF-7 and MCF-7/5C cells before and after different time points of treatment with E_2. We isolated proteins by anti-AIB1 or anti-pY immunoprecipitation (IP) and subsequent mass spectrometry (MS) identification as well as analysis of posttranslational modifications. Table 1 shows a summary of the number of proteins identified under different conditions and from repeat experiments. We identified a total of 1,667 proteins under different treatments and from the two cell lines studied. As mentioned above, experimental details and some of the initial data obtained were included in the past reports. From this complete set of proteins we filtered a subset for further analysis based on the following criteria. 1) Proteins with MS confidence intervals (CI) values smaller than 90% were removed to avoid false-positive results; 2) Proteins described to be non-specific interactors *e.g.* HSPA5 and Desmoplakin [Han et al., 2006] were removed; 3) High abundant, non-specific proteins *e.g.* keratins were removed; 4) Proteins identified at higher apparent MW on the SDS gels than the calculated, experimentally described or predicted molecular mass were removed. 5) Proteins that were identified at least twice in different experiments or from repeat experiments were included for analysis. These stringent filtering steps resulted in a subset of 165 proteins that either interacted with AIB1 (n=90) or were found as phosphotyrosine (pY)-complexed proteins (n=75).

Table 1. **Number of proteins identified from immunoprecipitation and mass spectrometry in MCF-7 and MCF-7/5C cells**. Numbers in parenthesis are the number of proteins identified at a confidence interval of >90% from the mass spectrometry, and their percentage of total proteins identified at all confidence levels.

Cell Type	**E_2**	**AIB1-IP**	**pY-IP**
MCF-7	-	321 (49, 15.2%)	172 (45, 26.2%)
	+	395 (79, 20%)	219 (44, 20%)
MCF-7/5C	-	281 (36, 12.8%)	204 (38, 18.6%)
	+	288 (54, 18.8%)	252 (46, 18.3%)
Total		**1001 (145, 14.5%)**	**666 (125, 18.8%)**

The Venn diagrams in Fig. 1 (A and B) show some overlap amongst groups for both AIB1 interacting and pY-complexed proteins. Comparisons were also made for the proteins between E_2-treated and untreated cells regardless of cell type, or between MCF-7 and MCF-7/5C cells regardless of treatment (Fig. 1, C and D). From a comparison of the different subsets shown in Fig. 1, we found more AIB1-interacting proteins in E_2 treated versus untreated cells in either MCF-7 or MCF-7/5C. This was not found for pY-complexed proteins in either cell line. Fig. 1 also shows that fewer AIB1 interacting proteins were identified from MCF-7/5C cells than from the parent MCF-7 cells in either E_2-treated or -untreated conditions. This was not as apparent for pY-complexed proteins in either cell line. This global initial analysis suggests that 1) AIB1-mediated protein-protein interactions are responsive to E_2 treatment and new protein complexes are induced by E_2; 2) at baseline the majority (9 of 16) of the proteins complexed with AIB1 in MCF-7/5C cells overlaps with proteins also present in AIB1 complexes in parent MCF-7 cells; 3) after E_2 the overlap between the cell lines is reduced to ¼ (8 of 32). Thus, the drivers of phenotypic differences in the E_2 response between MCF-7 and MCF-7/5C cells are likely revealed at the initial signaling level by distinct AIB1 protein-protein interactions; 3) Different pathways activated by E_2 also give rise to different sets of pY-protein complexes in both MCF-7 and MCF-7/5C cells, although the baseline comparison showed less overlap when compared to the AIB1 protein complexes (Fig. 1A vs. 1B).

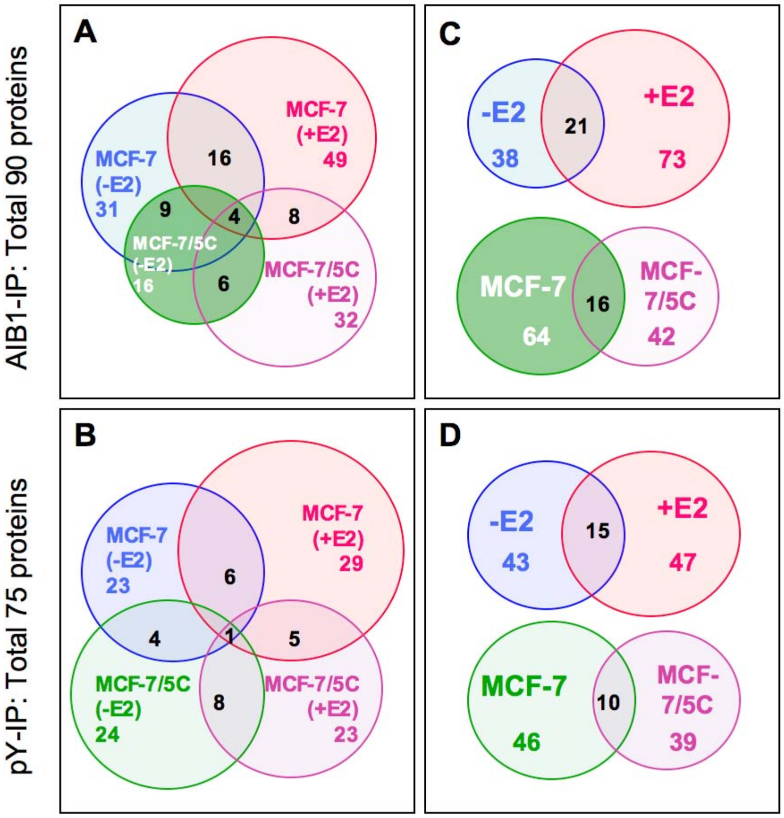

Figure 1. Venn diagrams showing the number of total and overlapping proteins identified from anti-AIB1-IP or anti-pY-IP experimental groups. Panels A and B shows AIB1-IP (A) or pY-IP (B) proteins from MCF-7 and MCF-7/5C, treated with or without E_2. Panel C and D shows overlapping proteins in combined AIB1-IP (C) or pY-IP (D) data sets.

Fig. 2 shows functional categories of the AIB1-associated (top) and pY complexed (bottom) proteins. Four major categories of AIB1-associated proteins were identified from MCF-7 and MCF-7/5C cells, *i.e.*, transcriptional regulation, signal transduction, metabolism, and cytoskeleton and structural proteins. In addition, the category of “chromosome and other ribonucleoproteins” is among the top-ranked (6th) ones in the AIB1 interacting protein group. When combined, the three categories of transcription regulation, signal transduction, and chromosome and ribonucleoproteins represent 31% of AIB1-associated proteins identified in this study, which is consistent with the broad role of AIB1 in cells. It is expected that some of these proteins may interact directly with AIB1, while others may associate indirectly with AIB1 within larger protein complexes. Although several proteins will still need to be validated for being authentic partners of AIB1 protein complexes, there is evidence for most of the proteins that they can participate in E_2-induced effects.

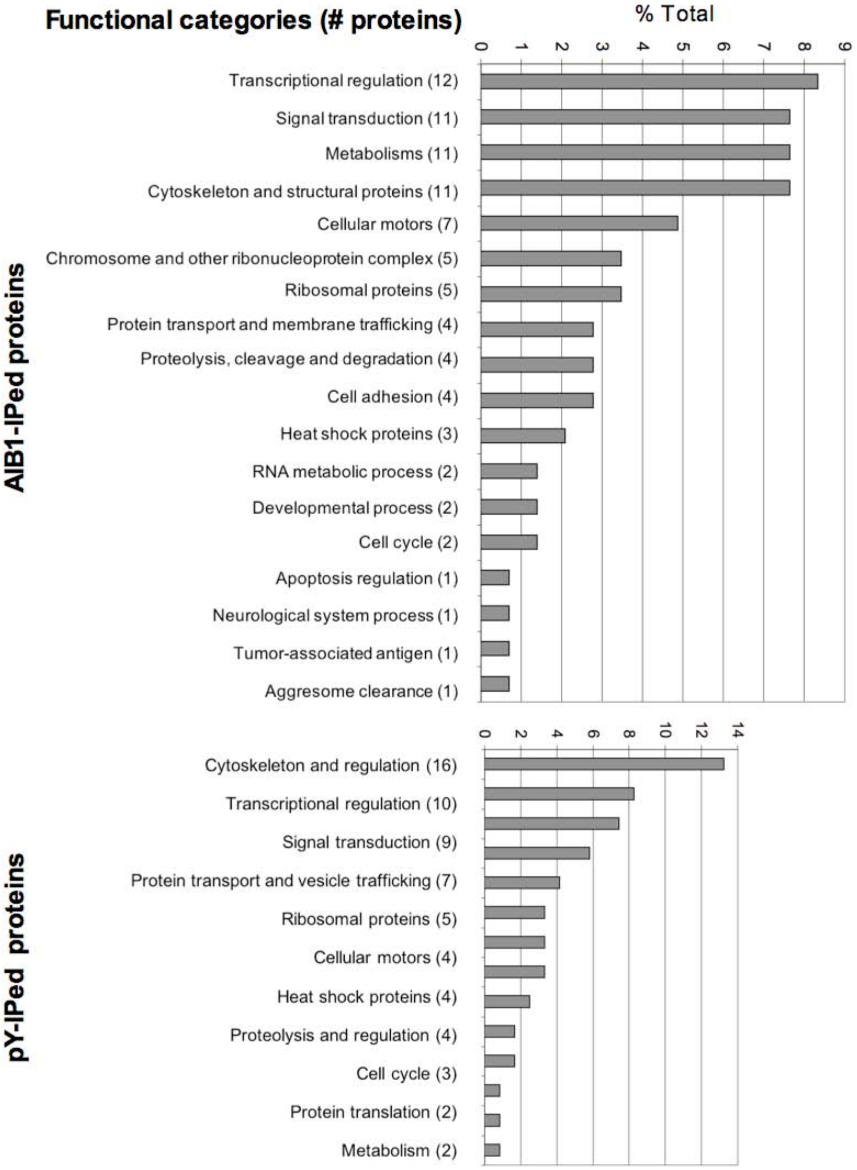

Figure 2. Gene Ontology (biological process) profiles of AIB1 or pY complexed proteins. All proteins with confidence intervals (CI) values >90% from mass spectrometry identified in MCF-7 and in MCF-7/5C cells are shown.

Fig. 2 (bottom) shows the functional categories of pY-complex proteins of which more than half fall into four major categories, *i.e.* cytoskeleton regulation, transcription, signal transduction, and protein transport and vesicle trafficking, of which three are similar to those of the AIB1-interacting proteins. This matches with global functions of pY-complexed proteins that will participate in signal transduction including cytoskeletal remodeling.

It is interesting to note the differences in the functional profiles between AIB1- and pY-complexed proteins. Both groups contain proteins related to transcriptional regulation, signal transduction, and cytoskeletal function as the most prominent categories. Distinct grouping was observed for metabolism-related proteins where the AIB1 complexes contained eleven different proteins and only two were found in the pY group. These AIB1-interacting metabolic enzymes mainly participate in sugar, fatty acid and amino acid metabolisms, *e.g.* GAPDH, phosphoglycerate mutase 4, very long-chain specific acyl-CoA dehydrogenase, fatty acid synthase, and maleylacetoacetate isomerase. Quite strikingly, all of these enzymes were identified in MCF-7/5C cells, while only a few of them were also identified in MCF-7 cells. The significance of these AIB1-associated metabolic enzymes is not yet clear but this is an intriguing finding because several nuclear receptors are known to regulate metabolism, such as PPAR, CAR, PXR and PGC-1α, and they are druggable. Beyond this, there were five AIB1-interacting proteins in the category of chromosome and ribonucleoprotein complex but none were found in the pY-complex protein group, which is consistent with the role of AIB1 as a transcriptional coactivator.

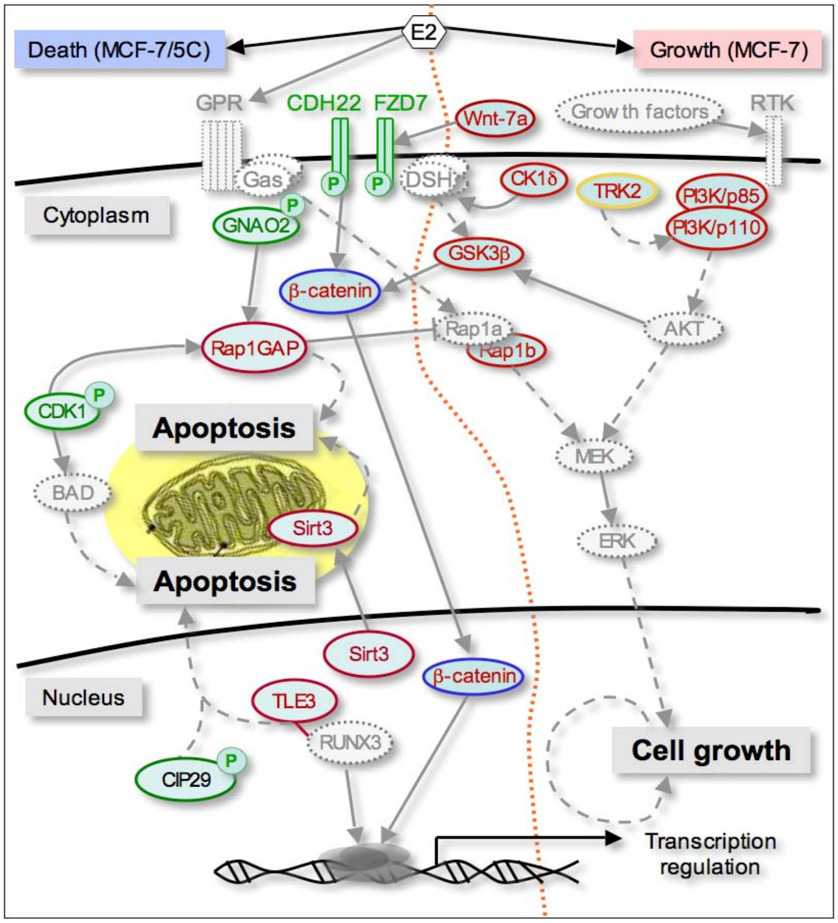

Figure. 3. Putative pathways involved in cell growth and apoptosis (death) in E2-treated MCF-7 versus MCF-7/5C cells. The dotted orange line roughly separates the pathways in MCF-7/5C (left, apoptosis) and MCF-7 cells (right, cell growth). Solid red line-circled are AIB1 and green line-circled are pY-interacting proteins (with "P" to indicate pY). Proteins in grey are those in known canonical pathways but not identified in the experimental series. The blue line-encircled protein (β-catenin) is identified in both treated and untreated MCF-7/5C cells, while the yellow-line encircled proteins are identified in both cells with or without E_2 treatment. Solid arrows indicate direct interactions (e.g. CDK1 phosphorylates Rap1GAP) or translocations (e.g. Sirt3) of proteins, while dashed arrows indicate indirect actions of proteins (e.g. AKT activate MEK through several steps). Inhibitory effects are indicated by hammer-headed lines, stimulatory effects by arrows.

Putative pathways linking to growth and apoptosis.

Based on the analysis results to date, we further refined our proposed pathway models. Here we propose a number of proteins, tyrosine-phosphorylated or interacting/associating, as involved in the growth or apoptosis pathways in E_2-treated MCF-7 and MCF-7/5C cells, respectively. Fig. 3 depicts putative pathways including GPCR, PI3K/AKT and Wnt/β-catenin that can lead to the mitochondria-mediated apoptosis or cellular growth. Three pY-IPed (Gα(o) or GNAO, CDK1 and CIP29) and three AIB1-IPed proteins (Rap1GAP, Sirt 3and TLE3) from E_2-treated MCF-7/5C cells are all linked to apoptosis, some of which are also known to interact with each other (Gα(o)-Rap1GAP and CDK1-Rap1GAP). Some of these proteins are nuclear, suggesting their dynamic movement either alone or complexed with AIB1 in response to different signals. PI3K catalytic and regulatory subunits were both AIB1-IPed in E_2 treated but not in untreated MCF-7 cells, which are key signaling proteins to activate the MEK/ERK signaling cascade through activation of AKT and lead to cellular growth. A non-receptor tyrosine kinase TRK2 was AIB1-IPed in both MCF-7 and MCF-7/5C cells with or with E_2 treatment, but it may be involved in PI3K/AKT signaling only in E_2 treated MCF-7 cells, as it is known to activate PI3K/p100 catalytic subunit. In addition, Rap1b was AIB1-IPed in E_2 treated MCF-7 cells, suggesting GPCR may also participate in the cell growth through MAPK signaling pathway.

In addition to the GPCR and PI3K/AKT pathway, there was a clear indication that Wnt/catenin pathway was affected in both MCF-7 and MCF-7/5C cells. Cross talks between Wnt and growth factor signaling pathways play important roles in many cancer processes such as progression and metastasis [Hasson et al., 2005, 2006]. It appears that Wnt/catenin pathway was activated in E_2 treated MCF-7/5C cells as shown by tyrosine-phosphorylated membrane receptors cadherin (CHD22) and frizzled (FZD7). In MCF-7 cell, Wnt ligand (Wnt-7a), CK1δ, and GSK3β were AIB1-IPed, which are known to modulate the Wnt signaling through phosphorylating the frizzled-induced DSH complex (by CK1) or β-catenin (by GSK3), respectively. It is unclear how Wnt signaling was involved in the E_2-induced growth or apoptosis of the breast cancer cells, but clearly multiple components in Wnt signaling interacted with AIB1 and responded to the E_2 treatment in the breast cancer cells.

Other complexes of AIB1 or tyrosine-phosphorylated proteins relevant for signaling

Some of the AIB1-interacting proteins in the category of “transcriptional regulation” were seen in both treated and untreated MCF-7 cells, but were only seen in E_2-treated and not in untreated MCF-7/5C cells, e.g. catenin delta-1, TLE-3, PRDM5. This suggests an altered AIB1-interaction pattern in MCF-7/5C cells and an increased response of those proteins to E_2 stimulation in the context of MCF-7/5C cells. Interestingly, TLE-3 can form transcriptional repressor complex with RUNX3 that is a known tumor suppressor and is invovled in apoptosis in gastric cancer [Nagahama et al., 2008], and PRDM5 has also been linked to cancer cell apoptosis [Deng and Huang, 2004]. The number of metabolism related AIB1-interacting proteins was significantly increased in response to E_2 in both cell lines and included proteins such as Sirt3 and peptide deformylase 3. In addition, AIB1-interacting proteins related to protein degradation/proteolysis were only seen in MCF-7/5C cells. In contrast, tyrosine-phosphorylated proteins of this functional category were seen in both MCF-7 and 5C cells, such as PSME2, PAPPA and ubiquilin-4.

Eighteen proteins were both AIB1- and pY-IPed from the two breast cancer cells, half of which are cytoskeletal and cellular motor proteins, others include those of signal transduction (calmodulin), transcription regulation (ZNF169), and cell cycle (ASPM). There are 22 AIB1-IPed proteins that are also part of the global AIB1 interaction network (see below), including proteins of signal transduction (*e.g.* camodulin), transcriptional regulation, cytoskeleton and heat shock. There are 18 pY-IPed proteins that are part of the global AIB1-interaction network, eight of which are also AIB1-IPed, suggesting that these proteins are likely to interact or associate with AIB1 as well as to be tyrosine phoshporylated.

Differential post-translational modifications of the estrogen receptor (ER)

In parallel with the above studies we have initiated a series of experiments to assess whether the ER shows differential posttranslational modification at baseline and after E_2 stimulation in MCF-7 versus MCF-7/5C cells. Such differences would indicate differential constitutive or E_2 activated pathways that modify the activity of the ER.

In an initial series we thus first established a reliable and effective method to isolate sufficient amounts of ER protein from cultured cells for posttranslational modification studies by LC-MS. The ER is a relatively low abundant cellular protein that can be identified by MS, although posttranslational modification studies are difficult because they require larger amounts of protein for analysis. After generating sufficient amounts of cell lysates in the Jordan laboratory (under Task 2a), we analysed the ER after immunoprecipitation. We found a total of 34 tentative distinct phosphorylation sites in the ER isolated from MCF-7 and MCF-7/5C cells without and with E_2 stimulation. These sites include several well-known sites such as S104/106, S118, Y537 as well as a number of unknown phosphosites or ones only noted in few technical reports. E_2-induced phosphorylation at ERα-S118 was also validated by immnoblot analysis using a phospho-specific antibody (Task 2a, Fig.1). At least three of the phosphorylation sites show reliable differential phosphorylation when comparing MCF-7 and MCF-7/5C cells. Although, it will be necessary to confirm these initial findings, we will be able to mine the extensive literature that describes the modifying pathways and connect that information with the findings already in hand.

Global AIB-1 interaction networks.

We searched PubMed database for all literature related to AIB1 since its discovery in 1997. A total of ~650 papers (as of June 2009) were retrieved, of which ~250 papers were curated that contain AIB1 interaction or functional association information, and a total of 91 AIB1 interaction partners were obtained and used as the seeds for the interaction network. The 91 proteins are of several major categories including transcription, cell communication, developmental process, cell cycle, etc.

The annotated protein-protein-interaction (PPI) data in existing bioinformatics databases were obtained from IntAct, which contains high throughput PPI data from two-hybrid and immunoprecipitation in addition to literature data [Kerrien et al., 2007]. The AIB1 interaction networks were constructed based on the binary interactions of the curated 91 AIB1 interacting proteins and those from the PPI database [Hu et al., 2009]. The network was clustered and filtered, and major hubs were selected and displayed with Cytoscape for visual examinations. The network is composed of ~1150 proteins, including 21 highly connected nodes as major hubs (Fig. 4), including p53, BRCA1, BCL2, ABL1, CDK2, CDK4, EGFR, ER (=ESR1), p38, and MYC.

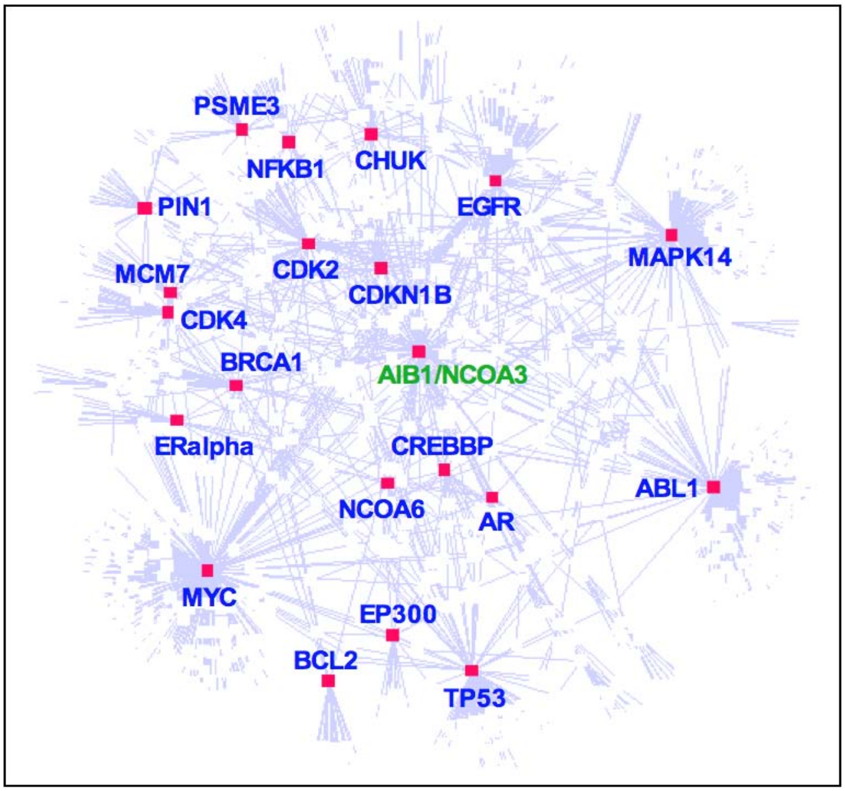

Figure 4. A global AIB1 interaction network showing the major hub proteins. Twenty-one hubs (red square and labeled with gene symbols) are identified using a cutoff of a node degree of 20.

The AIB1 interaction network analysis corroborates the broad roles of AIB1, such as close associations with oncogenesis, cell cycle regulation, and proteosome-related protein degradation. For example, PSME3 is a proteasome activator involved in the degradation of several proteins that regulate cell cycle, transcription, and the maintenance of chromosomal stability [Zannini et al., 2009]. Interestingly, we identified PSME2, one of the activators of the 20S proteasome, as Tyr-phosphorylated proteins in E_2 treated MCF-7 and untreated MCF-7/5C cells, suggesting that it may play a role in the AIB1-mediated signaling and the cellular responses. Several commonly IPed AIB1 network proteins are related to cytoskeleton and microtubule, suggesting that they are involved in the dynamic assembly and trafficking of AIB1 protein complexes in response to E_2 treatment. In summary, the AIB1 interaction network allows for a close examination of its functional association with other genes and may aid in the formulation of hypothesis on its potential roles in E_2-induced growth and apoptosis of breast cancer cells.

KEY FINDINGS

- We have completed our proteomics analysis to identify AIB1 and pY-complex proteins that are regulated differentially in response to E_2 in MCF-7 versus MCF-7/5C cells.

- We have built a pathway model that integrates this information into the differential response of these cells to E_2 (growth vs. apoptosis) and can now interrogate the relative contribution of these pathways revealed by the initial analysis.

- We have built an AIB1 interaction network that allows us to find functional association with pathways that have druggable targets.

- We have established MS analysis of the ER and found differential posttranslational modifications in the ER when comparing MCF-7 and MCF-7/5C cells. This should reveal additional signaling mechanisms that impact on ER activity and will be connected to the findings already in place.

- The findings reported above have been compiled for publication.

Future Directions

- Search for druggable targets along the pathways that control E_2 induced apoptosis will the focus of the next phase of studies.

- Analysis of posttranslational modifications of ERα as well as the controlling pathways will be one area of study.

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TASK 4: (TGen/Cunliffe) - To analyze E_2-induced survival and apoptotic pathways using gene arrays and siRNAs.

Task 4a: (Cunliffe) - Catalogue the transcriptional response using array-based expression profiling.

Task 4b: (Balagurunathan, Kim, and Cunliffe) - Identify regulatory networks for pathways indicative of differential responses to E_2.

Task 4c: (Azorsa and Cunliffe) - Interrogate pathways of endocrine resistance using high throughput RNA interference (HT-RNAi)

Tasks 4a and 4b efforts to categorize transcriptional responses and analyze the gene expression profiles determined by microarray hybridizations were carried out in parallel at the Translational Genomics Research Institute TGen) and at the Fox Chase Cancer Center (FCCC) sites.

Here we report work completed on Tasks 4a - 4c at the Translational Genomics Research Institute (TGen) site during year 3 of this COE.

Studies carried out at TGen in the laboratory of Heather Cunliffe PhD, with support from Yoganand Balagurunathan MS, Seungchan Kim PhD, and the laboratory of David Azorsa PhD.

MICROARRAY-BASED EXPRESSION PROFILING AND HIGH THROUGHPUT SIRNA SCREENING OF *IN VITRO* MODELS TO IDENTIFY GENES AND PATHWAYS ASSOCIATED WITH SURVIVAL AND APOPTOSIS MECHANISMS

Prior Progress - Tasks 4a and 4b.

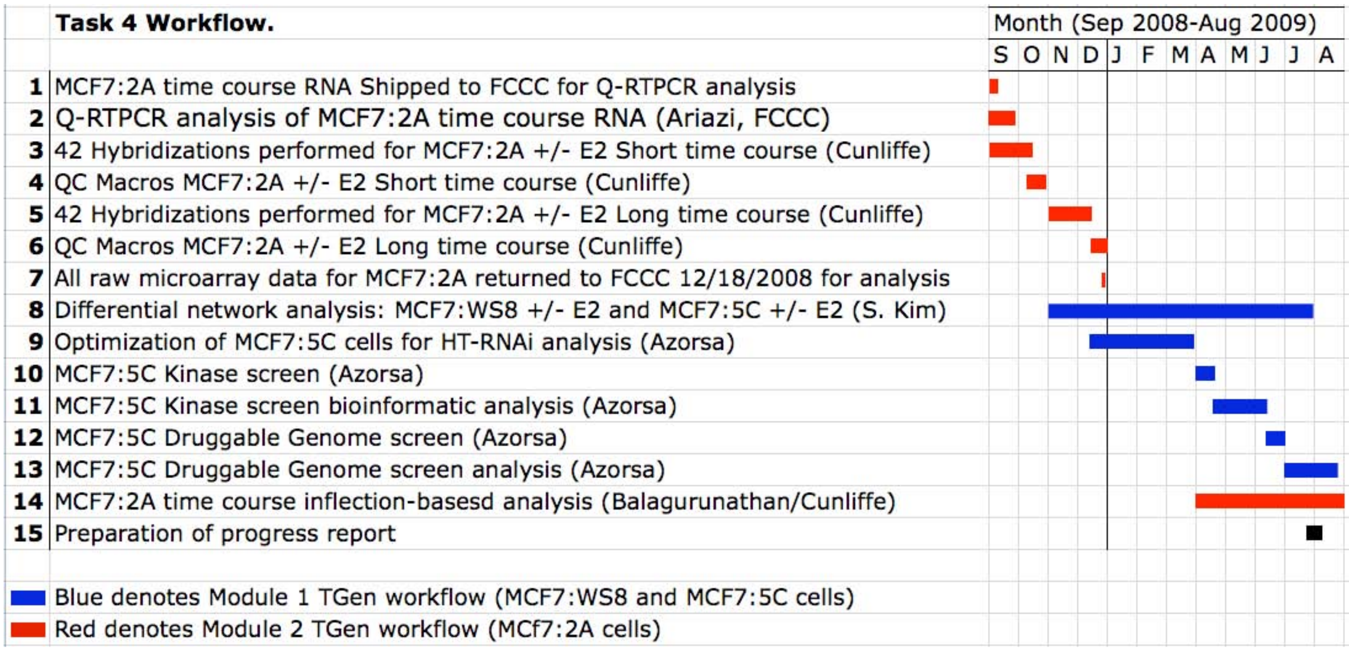
The overarching goal is to analyze global patterns of E_2-mediated gene regulation in wild-type (MCF-7:WS8) compared to phase II (advanced) models of endocrine resistance which undergo apoptosis following exposure to E_2. In prior years, gene expression profiling on the first module (including MCF-7:WS8 and MCF-7:5C cells with and without estrogen induction) were completed. This included time points of 2h, 6h, 12h, 24h, 48h, 72, and 96h with 6 replicates at each time point collected with no E_2, and 6 replicates collected at each time point following E_2 induction. Eighty four individual RNA extractions were performed for the MCF-7:WS8 time course and 84 from the MCF-7:5C time course. Forty two separate 2-color gene expression microarrays were performed using 44K oligonucleotide gene expression microarrays (Agilent Technologies) for the MCF-7:WS8 time course (using time point-matched RNAs as reference samples). The same procedure was followed for the MCF-7:5C cells, for the same time course (2h, 6h, 12h, 24h, 48h, 72, and 96h with 6 replicates at each time point collected). All data was returned to the Jordan Laboratory for pathway analysis. We also examined the gene expression by developing 3 custom methods that were Template-based, Distance-based, and Inflection-based as described in the Year 2 progress report.

Prior Progress - Task 4c

During prior years, we optimized the siRNA transfection methods and the growth conditions of MCF-7:5C and MCF-7:2A cells under the high-throughput assay conditions.

WORK ACCOMPLISHED - Task 4, Year 3.

The chart below summarizes the work performed on cell line Modules 1 and 2 as outlined in the original proposal, for Tasks 4a-c.



	Task 4 Workflow.
1	MCF7:2A time course RNA Shipped to FCCC for Q-RTPCR analysis
2	Q-RTPCR analysis of MCF7:2A time course RNA (Ariazi, FCCC)
3	42 Hybridizations performed for MCF7:2A +/- E2 Short time course (Cunliffe)
4	QC Macros MCF7:2A +/- E2 Short time course (Cunliffe)
5	42 Hybridizations performed for MCF7:2A +/- E2 Long time course (Cunliffe)
6	QC Macros MCF7:2A +/- E2 Long time course (Cunliffe)
7	All raw microarray data for MCF7:2A returned to FCCC 12/18/2008 for analysis
8	Differential network analysis: MCF7:WS8 +/- E2 and MCF7:5C +/- E2 (S. Kim)
9	Optimization of MCF7:5C cells for HT-RNAi analysis (Azorsa)
10	MCF7:5C Kinase screen (Azorsa)
11	MCF7:5C Kinase screen bioinformatic analysis (Azorsa)
12	MCF7:5C Druggable Genome screen (Azorsa)
13	MCF7:5C Druggable Genome screen analysis (Azorsa)
14	MCF7:2A time course inflection-basesd analysis (Balagurunathan/Cunliffe)
15	Preparation of progress report

🟦 Blue denotes Module 1 TGen workflow (MCF7:WS8 and MCF7:5C cells)

🟥 Red denotes Module 2 TGen workflow (MCf7:2A cells)

Table 1. Summary of work performed at TGen under Task 4a and 4b, September 2008-August 2009.

WORK ACCOMPLISHED - Task 4a.

Gene Expression Microarray Hybridizations (Heather Cunliffe, PhD).

Gene expression microarray time course analysis was conducted of the *in vitro* estrogen-responsive MCF-7:WS8 model, and the estrogen deprivation-resistant MCF-7:5C and MCF-7:2A models over a short-term time course (96 h). Additionally, a gene expression microarray study of the MCF-7:2A cells was conducted due to the observation that this cell line does not undergo apoptosis following E_2-stimulation as rapidly as the MCF-7:5C cells. E_2 induces apoptosis in the MCF-7:2A cells after approximately 7 days in culture. It was therefore necessary to extend the transcriptional analysis to capture genomic responses surrounding this phenotypic change.

As per **step 1** in the workflow chart above, RNA was prepared from MCF-7:2A cells treated with E_2 over a short and long time course in order to transcriptionally profile the response of this cell line to E_2 over the appropriate time frame needed for induction of apoptosis. 168 individual RNA extractions were performed by the Cunliffe Laboratory at TGen. This included 84 RNA extractions from the short time course (7 time points: 2h, 6h, 12h, 24h, 48h, 72, and 96h, 6 replicates per time point), both E_2 treated (42 RNA preps) and time point-matched non treated (42 RNA preps). Similarly, 84 RNA extractions were performed from the long time course (7 time points: 3, 4, 5, 6, 7, 8 and 9 days in culture, 6 replicates per time point, E_2 treated and non treated). All RNA isolations were quantitated and qualified prior to hybridization. An aliquot of all RNAs was returned to Dr. Eric Ariazi at the Fox Chase Cancer Center for verification of expected E_2-mediated expression response (workflow **step 2**). RNAs were then differentially labeled and hybridized using the same protocol and procedure as described for the MCF-7:WS8 and MCF-7:5C modules (workflow **steps 3-6.** See Year 2 progress report for additional detailed methodology). As with the other arrayed cell line modules, to reduce cross-experimental error due to the significant time frame in which these hybridizations needed to be batch hybridized, microarray profiling was performed such that at least one replicate from each time point was included in all batches of arrays. For example, the first replicate from all time points (2 through 96h) were hybridized in batch 1, etc. 83 of 84 hybridizations met array-based quality control statistics. The failed hybridization was replicate 1 of the MCF-7:2A 6-day E_2 treatment. The remaining 5 replicates from this time point were used for analysis. Raw data was returned to the Jordan Laboratory (workflow **step 7**), data was preprocessed by removing Q/C probes, and median intensity values were used for replicate array control probes. This reduced the raw data from 44,000 to ~41,000 features per array.

WORK ACCOMPLISHED - Task 4b-1.

Inflection-based Analysis (Yoganand Balagurunathan, MS).

In the Year 2 report, we described three discrete methodologies applied to interrogate the temporal gene expression data generated from MCF-7:WS8 and MCF-7:5C cells. The three temporal data analysis methodologies were as follows: 1) Template based, 2) Distance based, and 3) Inflection based. In Year 3, we have updated our Inflection-based method to include the MCF-7:2A cells gene expression profiles during the long-term time course (3 d – 9 d) to cover the time period during in which E_2-induced apoptosis occurs in these cells.

Inflection-based Temporal Gene Expression Data Analysis

A custom data analysis methodology was developed to identify individual driver or trigger (‘inflection’) genes that show a dramatic change in expression at a time point that mirrors a dramatic change in cell biological behavior (such as the apoptotic response of MCF-7:5C cells at approximately 96 h post E_2-treatment. The delta differences are defined as the change in expression of a given gene between time points with the starting point serving as the initial reference. Genes identified by this method are also considered with respect to wild-type MCF-7:WS8 cells that do not show the same apoptotic response to E_2 at a given time point. Using $\log_2$ normalized values, delta differences are progressively computed across the entire time course for each cell line being interrogated. A gene at a time point is considered an ‘inflection’ if the delta difference is 3σ greater than the computed experimental variation. Each time interval for a given gene is assigned a flag of ‘1’ if inflected otherwise a zero “0” to facilitate hypothesis generation and prioritization for subsequent validation.

$$D = [\text{Delta}G_i(\text{With-}E_2) - \text{Delta}G_j(\text{No-}E_2)]$$

$$\Rightarrow deltaD = {}^{1}\!/_{n_t} \sum_{k=1}^{n_t} DeltaG_{i:With-E2}(k) - DeltaG_{i:No-E2}(k)$$

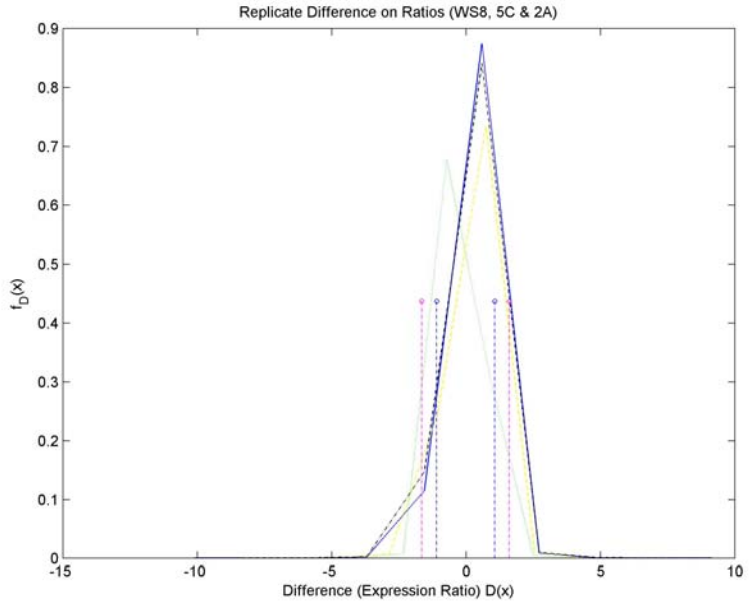


Figure 1. Distribution of differences among all possible experimental replicates within each group (blue solid line) with 1σ & 2σ cutoff lines in blue and red respectively. Positive and negative inflection gene distributions are denoted by the red and green dotted lines.

Finding Inflected Genes.

To briefly describe the methodology, the $\log_2$ normalized data is first converted to a level difference data by progressively computing the expression difference from its previous time observation. We refer to this as ‘delta’ data for 1 to 7 time points. For the first interval in which the starting time point does not have a predecessor set of baseline values, zeros were substituted for the difference ratio data. For the WS8 and 5C cells, the remaining time intervals were 6hrs – 2hrs, 12hrs – 6hr, 24hrs – 12hrs, 48hrs – 24hrs, 72hrs – 48hrs, 96hrs – 72hrs. For the MCF-7:2A cells, expression data were obtained over two independent time courses. Since ratio data was ‘delta’ transformed, the observation time points do not matter, and inflection measures could be cross compared between cell lines with different time point observations. In the 2A cells, E_2-induced apoptosis occurs with delayed kinetics; therefore the 2A long-term time course was examined. The time intervals for 2A cells were 3 days (reference substituted 0 ratios), 4 days – 3 days, 5 days – 4 days, 6 days – 5 days, 7 days – 6 days, 8 days – 7 days; and 9 days – 8 days. The delta transformation was repeated independently for all the replicates.

Our goal was to find inflections that are pronounced in all cell lines (WS8, 5C and 2A). Where a delta time point (1 to 7 meaning 2hrs to 96hrs in WS8 and 5C cells, or 3 days to 9 days in 2A cells) for a gene will be assigned ‘1’(inflected) otherwise ‘0’ (not inflected) only if the delta difference is greater than the confidence level set by the variation among the experimental replicates (Fig. 1). An inflection can occur in any time point but has to be limited to one of the experimental conditions. Lastly, genes showing inflections in any of the time points are sorted by total number of inflections (sum) across experiments in ascending fashion.

The same analysis as described above was repeated with ‘weighted delta data’ where outlier delta data points $(\mu\pm1\sigma)$ were omitted before computing the weighted average. This process results in identification of greater numbers of inflected genes. Tables 1 and 2 gives a frequency on the genes identified using delta difference and weighted delta difference, respectively. E_2-induced inflected genes primarily occur at one time point, suggesting strong and transient transcriptional flux at a discrete time-point. **Fig. 2** shows the top inflected gene specific to each cell line.

Table 1. Frequency of inflected genes using non-weighted delta difference data at a) 3σ, b) 2σ and c) 1σ levels Columns denoting number of inflections indicates genes inflected in 1, 2, 3 or ≥ 4 out of a possible 7 delta distance measures for each experiment.

(A) 3σ cutoff

Delta difference Data		**Number of Inflection (3σ cutoff)**				
	Experiment	**1**	**2**	**3**	**>4**	**Total**
1	5C	31				31
2	WS8	34	1			35
3	2A	22				22
4	5C & WS8	2				2
5	5C & 2A	0				0
6	WS8 & 2A	1				1
7	5C,WS8 & 2A	0				0
8	Any (5C,WS8, 2A; 5C&WS8; 5C&2A)	87	4			91

(B) 2σ cutoff

Delta difference Data		**Number of Inflection (2σ cutoff)**				
	Experiment	**1**	**2**	**3**	**>4**	**Total**
1	5C	160	8			168
2	WS8	297	10			307
3	2A	89	1			90
4	5C & WS8	34				34
5	5C & 2A	6				6
6	WS8 & 2A	10				10
7	5C,WS8 & 2A	2				2
8	Any (5C,WS8, 2A; 5C&WS8; 5C&2A)	546	66	5		617

(C) 1σ cutoff

Delta difference Data		**Number of Inflection (1σ cutoff)**				
	Experiment	**1**	**2**	**3**	**>4**	**Total**
1	5C	1031	83	11		1125
2	WS8	2514	297	22	2	2835
3	2A	756	33	1		790
4	5C & WS8	506				506
5	5C & 2A	81				81
6	WS8 & 2A	290				290
7	5C,WS8 & 2A	74				74
8	Any (5C,WS8, 2A; 5C&WS8; 5C&2A)	4301	1002	263	135	5701

Table 2. Frequency of inflected genes using weighted delta difference data at a) 3σ, b) 2 σ and c) 1 σ levels Columns denoting number of inflections indicates genes inflected in 1, 2, 3 or ≥ 4 out of a possible 7 delta distance measures for each experiment.

(A) 3σ cutoff

Weighted-Delta difference		Number of Inflection (3σ cutoff)				
	Experiment	**1**	**2**	**3**	**>4**	**Total**
1	5C	161	8	3		172
2	WS8	302	13	1		316
3	2A	641	81	6		728
4	5C & WS8	43				43
5	5C & 2A	23				23
6	WS8 & 2A	43				43
7	5C,WS8 & 2A	1				1
8	Any (5C,WS8, 2A; 5C&WS8; 5C&2A)	1296				1296

(B) 2σ cutoff

Weighted-Delta difference		Number of Inflection (2σ cutoff)				
	Experiment	**1**	**2**	**3**	**>4**	**Total**
1	5C	645	88	8		741
2	WS8	1665	245	24	2	1936
3	2A	2189	538	81	19	2827
4	5C & WS8	290				290
5	5C & 2A	486				486
6	WS8 & 2A	290				290
7	5C,WS8 & 2A	91				91
8	Any (5C,WS8, 2A; 5C&WS8; 5C&2A)	4499	1439	412	185	6535

(C) 1σ cutoff

Weighted-Delta difference		Number of Inflection (1σ cutoff)				
	Experiment	**1**	**2**	**3**	**>4**	**Total**
1	5C	1252	230	42	10	
2	WS8	2917	1002	266	62	
3	2A	5022	1938	633	241	
4	5C & WS8	2290				2290
5	5C & 2A	6713				6713
6	WS8 & 2A	2290				2290
7	5C,WS8 & 2A	5425				5425
8	Any (5C,WS8, 2A; 5C&WS8; 5C&2A)	9191	7552	5464	8680	30887

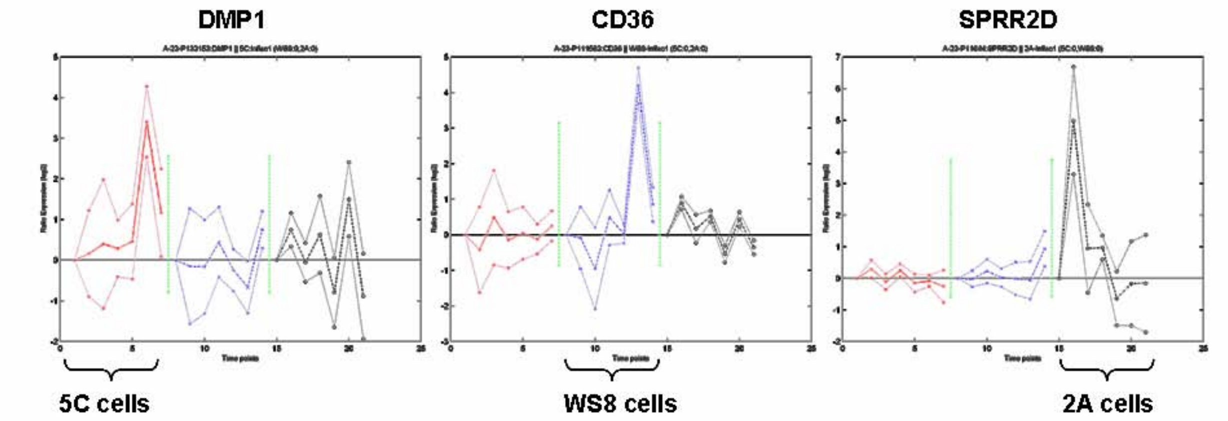


Fig. 2. Examples of top gene picks specific to each cell line as identified by the inflection-based methodology.

WORK ACCOMPLISHED - Task 4b-2.

Differential Network Analysis (Seungchan Kim, PhD).

Introduction

Dr. Seungchan Kim at TGen has begun modeling gene regulatory networks from genes with differential transcriptional behavior across MCF-7:WS8 and MCF-7:5C Breast Cancer Cell Lines. Over the past year, he has developed a procedure to build and compare regulatory networks of genes between these breast cancer cell lines, to specifically explore candidate pathways responsible for estrogen-induced apoptosis of MCF-7:5C cells. The key challenge addressed in this work is selecting a small set of genes that exhibit maximum differential transcriptional behavior and using them to build genetic regulatory networks. A probabilistic approach is used based on hidden Markov models in order to select the genes that exhibit the maximum amount of differential transcription. The expression profiles of selected genes are again modeled using hidden Markov models with adaptive number of states. Quantization of expression levels is performed by finding the most probable set of states for the data given the model, using the Viterbi algorithm. Two dynamic Bayesian networks, one for each cell line, are learned from the quantized gene expression data and they uncover the regulatory interactions among the selected genes.

Genetic Regulatory Networks (GRNs) describe the regulatory interactions that exist between genes at the cell level. Mathematically they can be represented as a distributed system with multiple inputs and outputs. The most refined level of descriptions involves the use of coupled differential equations that describe the various processes involved in the regulatory network [Chen 1999]. Though this approach can provide us with fine level of interactions, they do not scale well in representing a large regulatory network. Approaches based on clustering of genes using their expression levels are also used to group genes [Dhaeseleer 2000], the reasoning being that the genes sharing the same expression pattern are likely to be involved in the same regulatory process. However clustering techniques do not represent the regulatory interactions among genes to a sufficient level of detail.

The reverse engineering approach to inferring GRNs from expression data involves using network learning techniques. The simplest approach in this regard is to assume that the expression level of each gene is either 0(ON) or 1(OFF) – leading to the representation of GRNs as Boolean networks [Kauffmann 1993]. Incorporation of stochastic behavior in Boolean Networks results in the modeling of GRNs using Probabilistic Boolean Networks (PBNs) [Shmulevich 2002]. These networks are rich enough to represent the regulation of one gene by the other, but they still lack the flexibility of modeling finer details, because of their fixed number of states. Using Bayesian Networks (BNs) to analyze the expression data examines the conditional dependence and independence of the data [Friedman 2000]. One of the most elaborate procedures for modeling GRNs from time course microarray data has been performed using Dynamic Bayesian Networks (DBNs) [Murphy 1999]. They provide a significant extension to the paradigm of Bayesian Networks (BNs) by incorporating time information and hence they are naturally suited to modeling GRNs. The nodes of the DBNs represent the genes and arrows indicate the presence of regulation. Presence of regulation from gene to the other also implies conditional dependence between the nodes in the DBN, whereas absence of regulation implies conditional independence. When representing GRNs using DBNs, learning the regulatory interactions is equivalent to learning the structure of the DBN. When the gene expression profiles are modeled using linear differential equations, learning the GRN can be performed using a Kalman filter model [Perrin 2003]. However, in general learning the structure of DBN is performed either using greedy hill climbing or Markov Chain Monte Carlo (MCMC) methods [Wu 2008]. In our analysis, we use the MCMC approach though it is computationally intensive, as it is proven to be more robust.

Although DBNs are a general and powerful way of representing GRNs, learning the structure of DBNs from microarray data with large number of probes is a computationally intensive procedure. Therefore, it is necessary to reduce the number of nodes we deal with by preprocessing the expression data. Excluding the irrelevant genes, *i.e.* genes that do not show significant variation in their expression levels throughout the experiment is an effective way of preprocessing. Another method adopted to reduce the number of genes, is to cluster the genes that show similar changes in expression levels throughout the experiment. However, it should also be noted that in order to obtain useful results, preprocessing should be made specific to the case that is addressed. We are interested in examining the global Gene Expression Profiles (GEPs) of MCF-7: 5C cells compared to the MCF-7:WS8 cells. Therefore, to infer a GRN, we should use the genes with maximum differential transcriptional behavior across both the cell lines. We fit Hidden Markov Models (HMMs) to each gene expression profile in a particular cell line. Hidden Markov Models are a class of statistical models that can be used to modeling the time series data where the data at a particular time instance is statistically dependent only on the data in the previous time instance [Rabiner 1989]. The genes are then ranked by the amount of mismatch a GEP in a particular cell line has with the model of the GEP in the other cell line. Maximum mismatch corresponds to maximum differential transcription. When modeling the GEP using HMMs, we also take into account the replicate information to improve the robustness of the fit. The HMM model for the GEP provides a robust setup for finding the genes with maximum variation across the cell lines. Though there are approaches that model the GEPs using HMMs [Zeng 2006], the idea of learning HMMs taking the replicate information into consideration and utilizing the HMMs to identify genes with maximum differential transcription is novel.

Quantization of GEPs is required in order to learn discrete DBNs. Most of the quantization algorithms usually quantized the GEP into two discrete states inspired by the Boolean network approach [Shmulevich2002, Zhou 2003]. A more sophisticated approach of quantization allowing for adaptive number of states using mixture models has been proposed in [Chung 2006]. However, the disadvantage with these approaches is that they do not consider the time information in the GEP. In this paper, we propose a novel adaptive quantization procedure using HMMs where the maximum number of states is three (fixed), whereas the actual number of states for each GEP is variable. We achieve this by learning a HMM model for each GEP, adaptively determining the best number of states using a constraint on the means of the Gaussians representing each state. After learning the HMM, quantization is performed by determining the best state sequence for the data given the model using the Viterbi algorithm. The Viterbi algorithm is also modified to take the replicate information into consideration during the quantization process.

The aim of our novel approach is to identify the significant differences in regulatory mechanisms of genes in the wild type MCF-7:WS8 cells and MCF-7:5C cell lines, using their global expression data. This will reveal the genes and pathways that may be responsible for estrogen induced death of MCF-

7:5C cells. We find the genes with maximum differential transcription across the two cell lines by modeling their GEPs using HMMs with fixed number of states. HMMs for GEPs of selected genes are learned once again, using adaptive number of states and quantization is performed using the Viterbi algorithm. The quantized GEPs are used to learn one DBN per cell line that represent the underlying regulatory interactions among the selected genes. The differences in regulatory mechanisms are then inferred by comparing the two GRNs.

Methods

The complete gene expression dataset for each cell line can be represented in a three-dimensional matrix $\mathbf{G} = [g_{n,t,r}]$, where $n = 1,\ldots,N$ indexes the probes, $t = 1,\ldots,T$ indexes the time points and $r = 1,\ldots,R$ indexes the replicates. The GEP matrix of MCF-7: WS8 is indicated by the matrix $\mathbf{G}^1$ and the GEP matrix of MCF-7: WS8 is indicated by the matrix $\mathbf{G}^2$. Note that the GEP of each gene is still a single time series, though each time point has multiple replicates. The Bayes Net Toolbox (BNT) written in MATLAB by Kevin Murphy was adapted and used for implementing the steps in the algorithm that are related to HMMs [BNT_Murphy]. DBN structure learning was performed using DBmcmc MATLAB toolbox developed by Dirk Husmeier [DBmcmc_Husmeier]. The proposed algorithm was implemented in MATLAB and tested in an 8-core desktop PC running MS Windows XP.

Fitting HMMs to GEPs. Hidden Markov Models are robust statistical models for time series data that characterize the source that generated the data compactly. Hence, HMMs can be used for source modeling, prediction and classification. In our case, we fit one HMM per GEP of a dataset. Details on learning the HMM model are given in [Rabiner1989]. We will provide only the basic details and the modification that is needed in the setup to handle the replicates. Dropping the index *n*, we denote the GEP for a particular probe using $g_{t,r}$ where *t* is the index of the time point and *r* is the replicate index. In general, HMM learning needs multiple observations, but we have only a single observation with multiple replicates.

We fix the number of states to be three and they are given by $S = \{S_1, S_2, S_3\}$. We do not use adaptive number of states here because we use the HMMs learned here for ranking the genes. In that case, using different number of states for different GEPs will lead to difference in model complexity across the GEPs, which will render the direct comparison of the mismatch score across all the genes inaccurate. The probability of assigning state *i* at time instant *t* is given by $p(q_t = S_i)$. The observation at time *t* is given by $g_t = \{g_{t,r}\}_{r=1}^{R}$ and the observation at all time instants is given by $g = \{\{g_{t,r}\}_{r=1}^{R}\}_{t=1}^{T}$. We assume the observation at a particular time instant to be a following a mixture of Gaussians distribution, each state representing one Gaussian. Therefore the observation probability at a particular time instance and a particular replicate given a state is denoted by,

$$b_i(g_{t,r}) = p(g_{t,r} \mid q_t = S_i) = \mathrm{N}\ (g_{t,r}; \mu_i, \sigma_i^2)$$

where μ_i and σ_i^2 are the mean and variance of the ith Gaussian in the mixture respectively. The total observation probability for a time instance, assuming that the replicates at a particular instance are independent of each other, is given as

$$b_i(g_t) = \prod_{r=1}^{R} b_i(g_{t,r}) .$$

The HMM learning consists of using the Expectation Maximization algorithm to learn the parameter set Θ of the HMM, which are: the initial probabilities $p(q_1 = S_i)$, the transition probabilities $p(q_{t+1} = S_j \mid q_t = S_i)$ and the means and variances $\{\mu_i, \sigma_i^2\}$ of the Gaussians. The standard *forward-*

backward algorithm can be used with the observation probability given by $b_i(g_t)$ in order to learn the HMMs.

Ranking the Genes. Ranking of the genes is performed in order to find the genes with maximum differential transcription across MCF-7:WS8 and MCF-7:5C data sets. Statistically, this is equivalent to finding the genes whose GEP models in one dataset have the maximum mismatch with the GEP data in the other dataset. Let us the GEP model and data for a particular gene in MCF-7:WS8 dataset by g_n^1 and Θ_n^1, and the GEP model and data for a particular gene in MCF-7:5C dataset by g_n^2 and Θ_n^2. The mismatch score is given by,

$C(n) = -\left(\log p(g_n^1 \mid \Theta_n^2) + \log p(g_n^2 \mid \Theta_n^1)\right)$.

Ranking is performed by sorting the scores in descending order and the sorted indices are accumulated in the set **U**. The likelihoods $\log p(g_n^1 \mid \Theta_n^2)$ and $\log p(g_n^2 \mid \Theta_n^1)$ can be computed using the forward-backward procedure in HMMs.

Finding the Consensus Set: Learning the HMMs using EM algorithm typically yields a local minimum solution that is sensitive to initialization. Hence, the HMMs learned in multiple trials could be different for the same GEP and this leads to a different ranking in each trial. Hence if *P* genes are required to learn the DBN, it becomes necessary to repeat the ranking process multiple times and find a set of genes that are top ranked in all the trials. Suppose the sorted indices for each trial τ are placed in the set $\mathbf{U}^\tau$ and $\mathbf{U}_c = \bigcap_{\tau=1}^{\Gamma} \mathbf{U}^\tau(1:l)$ is the consensus set obtained using the top *l* ranked genes in all the trials. To find *P* consensus genes, *l* should be adjusted until $|\mathbf{U}_c| = P$. In this study we fix $P = 100$.

Fitting HMMs using Adaptive Number of States. HMMs of GEPs used for ranking cannot be used for quantization because they have same number of states for all genes. It would be advantageous to operate with lesser number of states for genes which do not show much change in the expression levels over time. We fit HMMs again for the consensus set of genes using adaptive number of states, fixing the maximum states to be 3. The criteria for determining the states is that the mean values of the states, that show the mean expression levels must be at least $\log_2(1.5) = 0.5850$ apart. This is because; we require at least 1.5 fold change in expression level for it to be considered significant. We first fit a HMM with maximum number of states and reduce the number of states until the constraint on the mean expression levels are met. This is repeated for all *P* genes in the consensus set $\mathbf{U}_c$ and the parameters of the HMM are given by Θ_p^1 and Θ_p^2 for the two datasets where $p = \{1, ..., P\}$.

Quantizing the GEPs. Quantizing the GEPs is necessary in order to capture the key variations in the expression data and learning the discrete DBNs. The GEP of the pth gene in the consensus set is given by g_p and it has *T* time points and *R* replicates. The quantized GEP is given by $\hat{g}_p$ and it just has *T* time points and the replicate information is absorbed in the quantized value itself. The best state sequence $\hat{g}_p = \{q_{p,1}, q_{p,2}, ., q_{p,T}\}$ for the observation g_p given the HMM model Θ_p is computed using the Viterbi algorithm [Rabiner1989]. The observation probability for time instant *t* is modified to include the replicate information as $b_i(g_{p,t}) = \prod_{r=1}^{R} b_i(g_{p,t,r})$. This modification to observation probability to include replicate information leads to a robust estimation of the best state sequence, which is equivalent to the quantized GEP.

Learning the DBNs. The quantized gene expressions for each dataset are given by the matrix $\hat{G} = [\hat{g}_p]_{p=1}^{P}$, and they are used to learn the DBN for that particular cell line. Learning the DBN is equivalent to finding the structure M^* that maximizes the posterior probability $p(\mathrm{M} \mid \hat{G})$. The structure learning for DBN is performed using a MCMC procedure described in [Husmeier2003]. To restrict the complexity to a manageable level, the maximum number of possible regulating genes for a particular gene is fixed to 5. The MCMC proceeds over multiple iterations by modifying the network using a proposal move. The proposal move consists of adding, deleting or reversing an edge. The procedure uses the Metropolis-Hastings acceptance criterion [Hastings1970] to accept or reject the proposal move. Under fairly general conditions, this algorithm produces a Markov chain that converges to the true posterior distribution $p(\mathrm{M} \mid \hat{G})$. Typically, a large number of networks are generated using this procedure along with the posterior probability for each edge. A representative network structure can be obtained by including only the edges that have a posterior probability greater than a threshold. The structures of the learned DBNs are representatives of the regulatory interactions between the genes that exhibit maximum differential transcription across the two cell lines.

Preliminary Results

Gene Expression Dataset

Global gene expressions of MCF-7:WS8 and MCF-7:5C cell lines were measured using the Human Genome CGH microarray kit 44k of Agilent. The expressions were collected over 41000 probes over 7 time points – at 2, 6, 12, 24, 48, 72 and 96 hours. For each time point 6 replicates were collected for ensuring the statistical veracity. The datasets were converted to a $\log_2$ scale. The MCF-7:5C dataset had a replicate missing in the third and fifth time points. The missing replicates were imputed using the mean of other replicates in the same time points.

Learned Regulatory Networks

The regulatory networks learned for the two cell lines using the global gene expressions and the approach proposed above are given in Figs. 3 and 4. An edge between two nodes is shown only if the posterior edge probability is greater than 0.5. Fig. 3 gives the regulatory network learned for MCF-7: WS8 cell line with the top 100 genes exhibiting differential transcriptional behavior across the two cell lines. We do not show here, the genes that do not have any connection with other genes. So, only 36 genes out of the 100 selected genes are shown in Fig. 4. Fig. 5 gives the MCF-7:5C regulatory network learned for the same 100 selected genes. In this case only 40 genes out of the 100 selected genes are shown. The gene expression values were thresholded between 2 and -2, and color-coded with bright red indicating 2, black indicating 0 and bright green indicating -2.

The genes in the learned networks are referred by their genes symbols. Note that some genes in the networks do not have gene symbols and hence they are named using their probe IDs. They are color coded according to their mean expression value, computed after quantization, across all the time points. The common genes are differentiated in the two networks using an ellipse instead of a box for all the other nodes. It can be seen that 14 nodes are in common between the two networks and they are ATF5, LFNG, MXD1, DUSP5, EVL, COL6A1, RIN1, TFPT, WNT11, A_32_P18034, C3orf59, KCNF1, XBP1 and FABP5. Almost all of the common genes show clear difference in mean expression values except a few. This is because the mean expressions may be very close for two GEPs that are markedly different over time points.

Referring to the MCF-7: 5C network, several interesting observations are evident. The DAPK2 gene, responsible for the induction of apoptosis is over-expressed. Similarly the genes FABP2 - responsible for glucose transport and metabolism, CISH – responsible for activation of Protein Kinase C (PKC) and RIN1 – responsible for RAS/RAB signaling, are also over-expressed. Our learned network for MCF-7: 5C cell line has revealed a useful structure, where the mean value of expressions of the key genes clearly shows that apoptosis is in progress in an aggressive cell line. Further analysis of this network structure could lead to identification of the candidate pathways responsible for this behavior.

It is also interesting to study the network structure for the wild type MCF-7:WS8 cells. It can be seen that DAPK2 and CISH are absent in the learned network, which imply that induction of apoptosis and PKC activation are not present in the MCF-7:WS8 cell line, consistent with the known biologic behavior of these cells. Instead the ATF5 gene, which is responsible for the negative regulation of apoptosis, is over-expressed and the TFPT gene responsible for the positive regulation of apoptosis is under-expressed. This corroborates the fact that the MCF-7:WS8 cells are growing normally in the presence of estrogen. Furthermore, the genes TERT and XBP1 which pay key roles in DNA metabolism and cell cycle progression are also over-expressed, as expected. Other genes of interest in this network are PMP22, which leads to cell cycle control and PKIA, which causes Protein Kinase A (PKA) inhibition and they are both under-expressed.

Together, our preliminary observations indicate a clear distinction in the structure of MCF-7:WS8 and MCF-7:5C networks and the MCF-7:WS8 network can be used as a reference to study the behavior of the MCF-7:5C regulatory network under exposure to estrogen.

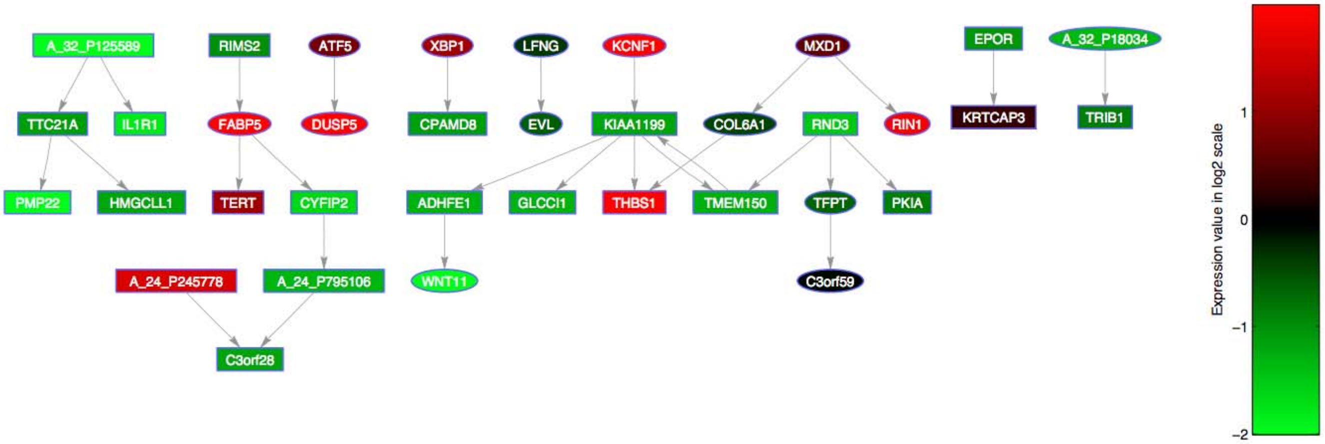

A_32_P125589
RIMS2
ATF5
XBP1
LFNG
KCNF1
MXD1
EPOR
A_32_P18034
TTC21A
IL1R1
FABP5
DUSP5
CPAMD8
EVL
KIAA1199
COL6A1
RND3
RIN1
KRTCAP3
TRIB1
PMP22
HMGCLL1
TERT
CYFIP2
ADHFE1
GLCCI1
THBS1
TMEM150
TFPT
PKIA
A_24_P245778
A_24_P795106
WNT11
C3orf59
C3orf28
Expression value in log2 scale
1
0
−1
−2

Figure 3. The GRN learned for the MCF-7:WS8 cell line for the genes that exhibit maximum differential transcription using the proposed approach in Figure 5. The gene expression values were thresholded between 2 and -2, with bright red indicating 2, black indicating 0 and bright green indicating -2. The elliptical nodes are common with the learned MCF-7:5C network. The color coding map is shown on the right.

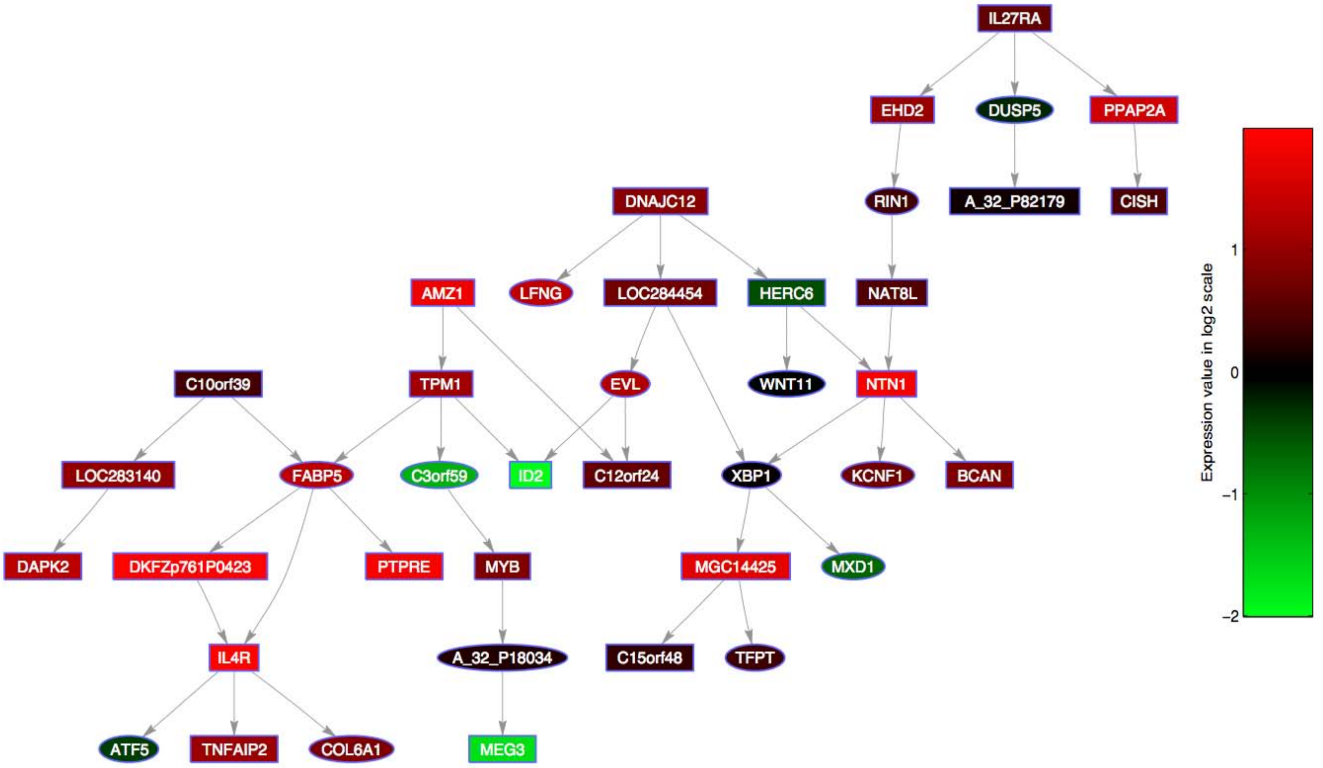

IL27RA
EHD2
DUSP5
PPAP2A
RIN1
A_32_P82179
CISH
DNAJC12
AMZ1
LFNG
LOC284454
HERC6
NAT8L
C10orf39
TPM1
EVL
WNT11
NTN1
LOC283140
FABP5
C3orf59
ID2
C12orf24
XBP1
KCNF1
BCAN
DAPK2
DKFZp761P0423
PTPRE
MYB
MGC14425
MXD1
IL4R
A_32_P18034
C15orf48
TFPT
ATF5
TNFAIP2
COL6A1
MEG3
Expression value in log2 scale
1
0
−1
−2

Figure 4. The GRN learned for the MCF-7:5C cell line for the genes that exhibit maximum differential transcription using the proposed approach in Fig. 5. The gene expression values were thresholded between 2 and -2, with bright red indicating 2, black indicating 0 and bright green indicating -2. The elliptical nodes are common with the learned MCF-7:WS8 network. The color coding map is shown on the right.

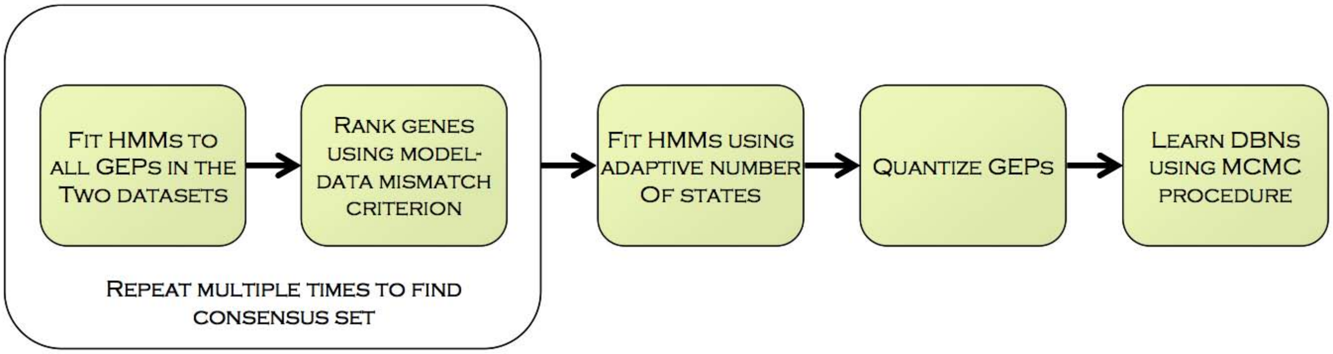

Fit HMMs to all GEPs in the Two datasets
Rank genes using model-data mismatch criterion
Repeat multiple times to find consensus set
Fit HMMs using adaptive number Of states
Quantize GEPs
Learn DBNs using MCMC procedure

Fig. 5. Illustration of the proposed approach to learn GRNs of genes that exhibit maximum differential transcription between MCF-7:WS8 and MCF-7:5C cell lines.

WORK ACCOMPLISHED – Task 4c

HIgh-throughput siRNA Screen of estrogen deprivation-resistant MCF-7:5C cells (David Azorsa, PhD)

Assay development for High-throughput RNAi (HT-RNAi) screening of the MCF-7:5C cell line was completed. MCF-7:5C cells have been screened using the human siRNA druggable genome library. This large-scale siRNA library consists of 28,000 siRNAs that target ~7,000 genes which are considered “druggable” as genes targeted by this library have characteristics that make them tractable targets for pharmacological inhibition. The library includes several categories of genes as defined by Gene Ontology annotations in Fig. 6.

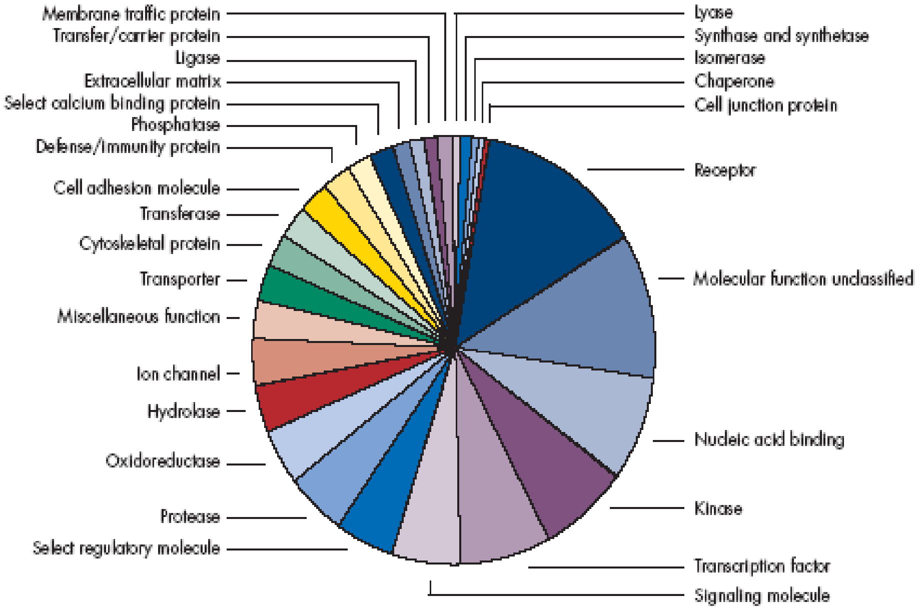


Figure 6. Gene classifications of targets from the Druggable Genome siRNA library

Two replicate siRNA screens have been performed on MCF-7:5C cells. For each experiment, duplicate 384-well plates of MCF-7:5C cells were transfected, and one of the duplicates was then treated with estrogen. In the siRNA experimental series with no estrogen, a number of siRNAs were identified to alter viability of MCF-7:5C cells. This data is represented as a cell viability waterfall plot (Fig. 7.). These genes are currently being compared to results from MCF-7 wild type cells to determine whether any genes are specifically associated with survival of MCF-7:5C cells.

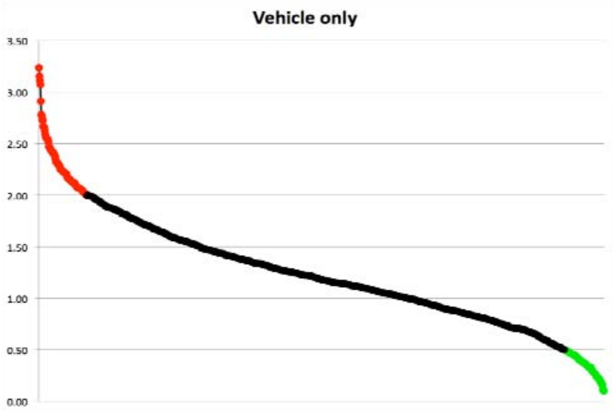


Figure 7. Waterfall plot of MCF7:5C cell viability. Red indicates transcripts whose siRNA-mediated downregulation increases cell viability $\geq$ 2 fold, and therefore have growth suppressive capacity in MCF7:5C cells. The majority of these genes are associated with cell metabolic behavior. Green indicates transcripts whose downregulation decreases cell viability $\geq$ 2 fold (termed by our laboratory as “Achilles heel genes”. The majority of these genes are known to be associated with cell cycle progression and survival, as would be expected.

In the siRNA experimental series with E_2, we identified a number of genes whose siRNA-mediated silencing was specifically associated with protection from E_2-mediated cell death, and did not affect cell viability in the absence of E_2. Output from one of the two screens is represented as a scatter plot of ratios (Fig. 8). The druggable genome consists of only 2 siRNAs per gene, and the statistically significant candidate ‘hits’ identified by this analysis were not always validated in both siRNAs. As is customary with respect to hit validation from the high throughput druggable genome screen, a custom "flexiplate" was purchased for the hit validation screen, and contains four separate siRNAs per gene. A small number of genes have been validated as positive for all four siRNAs from the flexiplate.

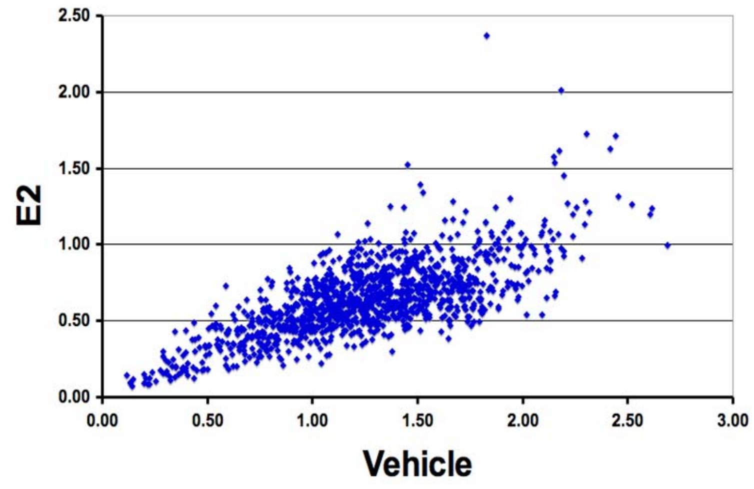


Figure 8. Scatter plot of ratio of cell viability comparing E_2-treated or vehicle-treated MCF7:5C cells following transfection with siRNA library. Transcripts whose siRNA-mediated downregulation does not reduce cell viability in the presence of E_2 relative to vehicle control are candidate genes likely to be associated with E2-induced apoptosis in MCF7:5C.

Key Research Accomplishments, Sept 2008-Aug 2009

- Completed gene expression profiling on MCF-7:2A cell line induced with estrogen over a short (96h) and long (9 day) time course. Data have been provided back to the CoE for joint analysis.

- Developed inflection-based analysis of genes expression profiles to identify genes that rapidly change in response to E_2. This analysis was applied to MCF-7:WS8, MCF-7:5C and MCF-7:2A (long-term time course) cells.

- Developed and applied differential network analytical methodology as a novel approach to characterizing pathways related to E_2-induced apoptosis in the MCF-7:5C cell line model.

- Completed high throughput RNAi screen of the MCF-7:5C and MCF-7 wild type cell lines. Successfully identified a small number of novel gene 'hits' that are protective of estrogen induced apoptosis. These candidates are currently being validated in "flexiplate" format at TGen in the Azorsa Laboratory.

- A manuscript describing genomic evolution of the long-term estrogen-deprived MCF-7:5C and MCF-7:2A cell line models is in preparation.

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TASK 4: (FCCC/Jordan) – To analyze E_2-induced survival and apoptotic pathways using gene arrays and siRNAs.

Task 4a: (Ariazi and Jordan) - Catalogue the transcriptional response using array-based expression profiling.

Task 4b: (Ariazi and Jordan) - Identify regulatory networks for pathways indicative of differential responses to E_2.

Tasks 4a and 4b efforts to categorize transcriptional responses and analyze the gene expression profiles determined by microarray hybridizations were carried out in parallel at the Translational Genomics Research Institute (TGen) and at the Fox Chase Cancer Center (FCCC) sites.

Here we report work completed on Tasks 4a and 4b at the FCCC site during year 3 of this COE.

Studies carried out at FCCC and led by Eric Ariazi PhD in Dr. Jordan's laboratory, with bioinformatic support from Michael J. Slifker MS, Karthik Devarajan PhD, Suraj Peri PhD, Yan Zhou PhD and Eric Ross PhD.

GENE EXPRESSION MICROARRAY ANALYSIS OF E_2-INDUCED APOPTOSIS OF ESTROGEN DEPRIVATION-RESISTANT MCF-7:5C AND MCF-7:2A CELLS COMPARED TO WILD-TYPE MCF-7:WS8 CELLS

Prior Progress

Wild-type WS8 cells respond to E_2 with robust growth, whereas the estrogen deprivation-resistant 5C and 2A cells undergo E_2-induced apoptosis; resistant 5C cells begin dyeing after 3-4 days of E_2 treatment and the 2A cell after 7 days. The growth responses of these cell lines to E_2 are shown Under Task 2b-2, Fig. 1.

During Years 1 - 2 of this award, RNA samples from all three cell lines were collected and purified. Also during Years 1 - 2, the RNA samples from WS8 and 5C cell were labeled and hybridized to gene expression microarrays hybridizations. The microarray hybridizations of RNA samples from 2A cells were carried out during the current Year 3 (see Task 4a:TGen/Cunliffe). A brief summary of the work accomplished in prior years follows here. WS8, 5C and 2A cells were treated plus/minus 10^{-9} M E_2 over a 96 h time course, with RNA collection time points at 2 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 96 h. This 96 h time course covers the period in which the 5C cells undergo E_2-induced apoptosis. To capture the period in which the 2A cells undergo E_2-induced apoptosis, a relatively long-term time course was conducted in which 2A cells were treated plus/minus 10^{-9} M E_2 and RNA was collected daily over 3 – 9 days. In all time courses, 6 replicate RNA samples were collected per group. A total of 4 time course experiments were carried out; 3 short-term 96 h time courses, one each in WS8, 5C and 2A cells, and 1 long-term 3-9 day time course in 2A cells. A total of 4 time courses × 7 time points/time course × 2 treatments/time point × 6 replicates/treatment for a total of 336 RNA samples (unpurified lysates) were collected. These RNA collection time course experiments were conducted at the Fox Chase Cancer Center during Years 1 and 2. The unpurified RNA lysates were barcoded and sent to TGen. At TGen in the Cunliffe laboratory, the RNA samples were purified, analyzed for integrity by RNA microfluidic electrophoresis, and an aliquot of each sample was returned to Fox Chase Cancer Center. At the Fox Chase Cancer Center, the purified RNA samples were quality controlled by measuring mRNA expression by real-time PCR of two E_2-inducible genes, c-Myc which shows induction by 2 h and then decreases, and pS2 which shows induction by 6 h and remain high for the remainder of the time course. The results of which samples showed the expected expression levels of c-Myc and pS2 were shared with

TGen. Only RNA samples that passed these quality control tests were hybridized to microarrays at TGen.

WORK ACCOMPLISHED – Task 4a.

Catalogue the transcriptional responses using array-based expression profiling.

Microarray Hybridizations

Gene expression microarray hybridizations of the estrogen deprivation-resistant MCF-7:2A cells lines over a short 2 – 96 h and a long 3 – 9 d time course were completed at TGen (for details see Task 4a: TGen/Cunliffe). All microarray experiments were carried out using the 2-color Agilent 4x44k human oligonucleotide platform. The microarrays were competitively hybridized with RNA isolated from 10^{-9} M E_2-treated cells against matched control-treated cells. The resulting microarray hybridization characteristics were quality controlled and the raw data were supplied to Fox Chase Cancer Center.

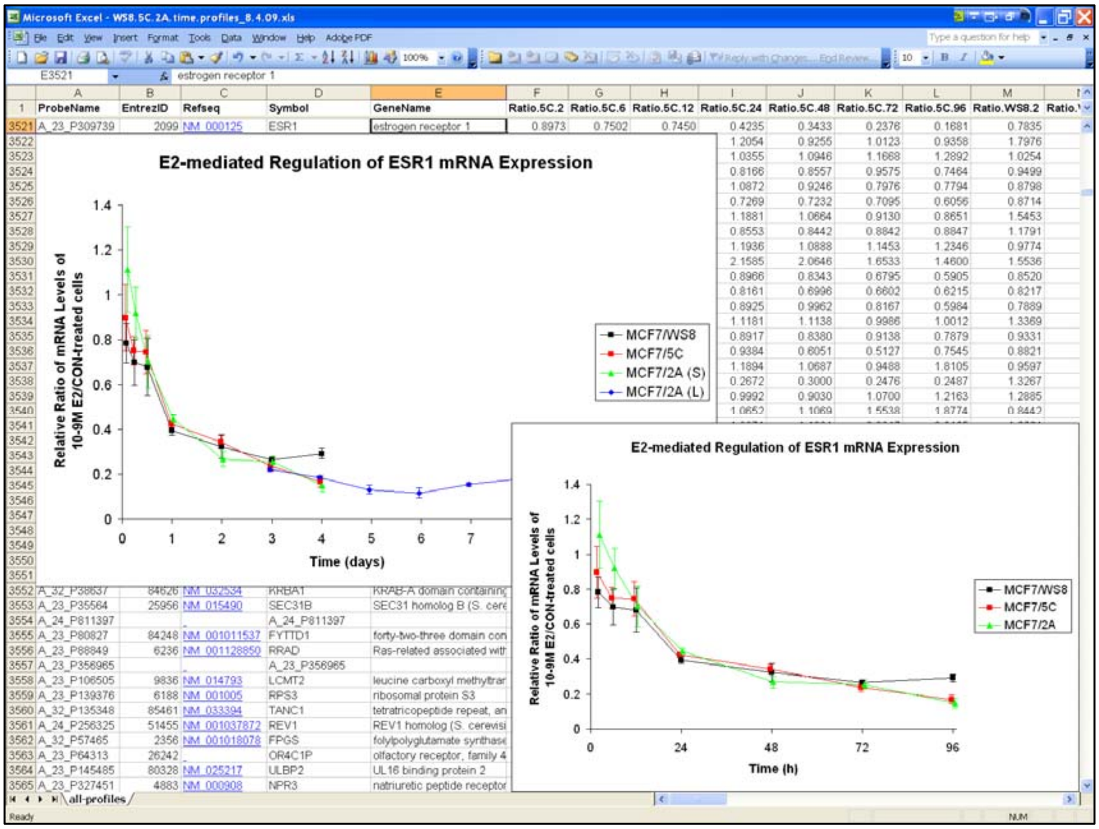
This completes all gene expression microarray experiments involving *in vitro* (cell line) models of antihormone resistance as originally proposed.

The emphasis now turns to bioinformatic analyses of the collected global gene expression profiles. Fox Chase Cancer Center and TGen are performing these analyses in parallel. The bioinformatic analyses being conducted at TGen are described under Task 4b:TGen/Cunliffe. Here we describe the bioinformatic analyses conducted at Fox Chase Cancer Center.

Automated Graphing of Expression Profiles

In Year 2, we implemented an Excel-based tool that automatically plots time-course gene expression profiles of any probe selected from the microarray experiments. During year 2, this database contained gene expression data from the MCF-7:WS8 and MCF-7:5C cells over the 96 h time courses. This database and automated graphing tool has been updated and improved in the current year 3 in a number of ways. The database has been updated to now contain the MCF-7:2A cell gene expression data over both the short-term 96 hour and the long-term 3 – 9 day time course. Two Visual Basic macros were written which allow the dynamic (‘on-the-fly’) creation of color-coded expression time-course profile plots for any selected probes together with error bars, on a linear scale. One macro displays profiles all four time courses, and the second displays profiles of the short-term data alone (Fig. 1). An additional two similar Visual Basic macros were prepared that display the profiles on a $\log_2$ scale (Fig. 2). These macros have proved very useful for quickly assessing interesting expression profiles. In addition to summary expression values and error estimates, the database includes appropriate significance measures for each probe in the database. To be precise, the empirical Bayes moderated one sample t-statistics was computed (as implemented in the R/Bioconductor package LIMMA [www.bioconductor.org, (1)]) for each probe in all cell lines and time points, based on the $\log_2$-ratios between E_2-treated samples and co-hybridized control samples.

A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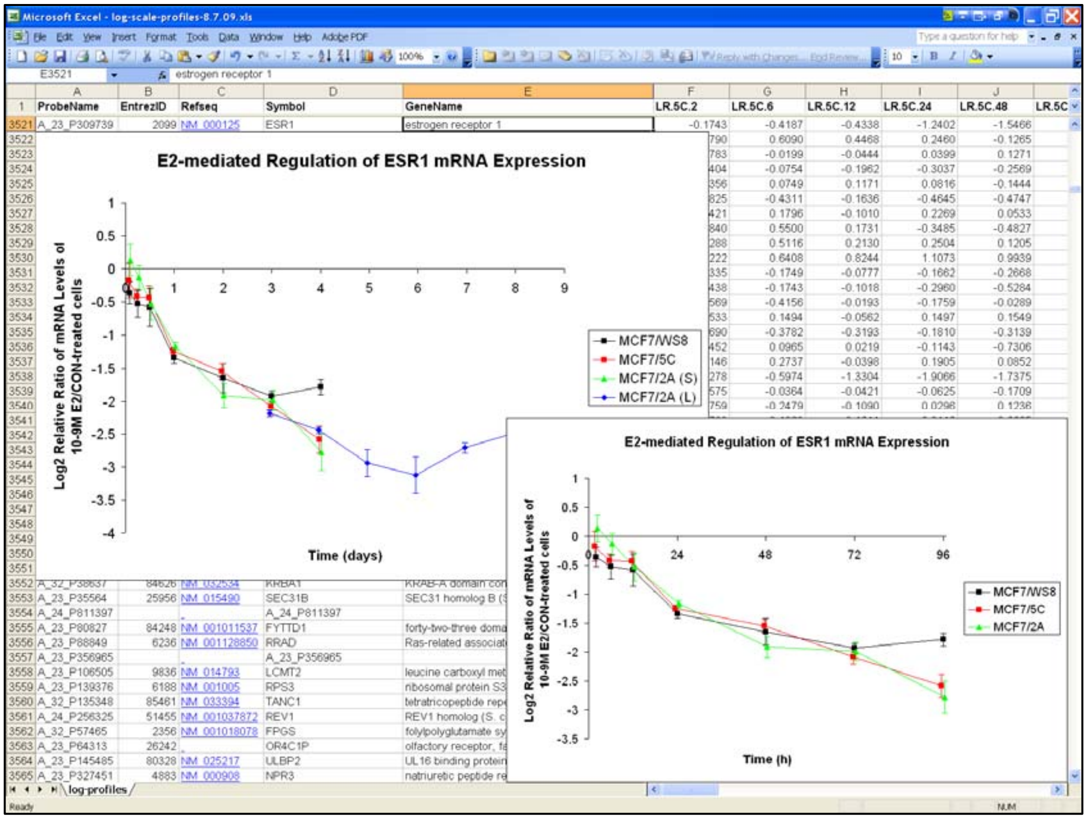
E3521
estrogen receptor 1
E2-mediated Regulation of ESR1 mRNA Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative Ratio of mRNA Levels of 10-9M E2/CON-treated cells
0
1
2
3
4
5
6
7
Time (days)
MCF7/WS8
MCF7/5C
MCF7/2A (S)
MCF7/2A (L)
E2-mediated Regulation of ESR1 mRNA Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative Ratio of mRNA Levels of 10-9M E2/CON-treated cells
0
24
48
72
96
Time (h)
MCF7/WS8
MCF7/5C
MCF7/2A
all-profiles
Ready
NUM

1	ProbeName	EntrezID	Refseq	Symbol	GeneName	Ratio.5C.2	Ratio.5C.6	Ratio.5C.12	Ratio.5C.24	Ratio.5C.48	Ratio.5C.72	Ratio.5C.96	Ratio.WS8.2	Ratio.'
3521	A_23_P309739	2099	NM_000125	ESR1	estrogen receptor 1	0.8973	0.7502	0.7450	0.4235	0.3433	0.2376	0.1681	0.7835	
3522									1.2054	0.9255	1.0123	0.9358	1.7976	
3523									1.0355	1.0946	1.1668	1.2892	1.0254	
3524									0.8166	0.8557	0.9575	0.7464	0.9499	
3525									1.0872	0.9246	0.7976	0.7794	0.8798	
3526									0.7269	0.7232	0.7095	0.6056	0.8714	
3527									1.1881	1.0664	0.9130	0.8651	1.5453	
3528									0.8553	0.8442	0.8842	0.8847	1.1791	
3529									1.1936	1.0888	1.1453	1.2346	0.9774	
3530									2.1585	2.0646	1.6533	1.4600	1.5536	
3531									0.8966	0.8343	0.6795	0.5905	0.8520	
3532									0.8161	0.6996	0.6602	0.6215	0.8217	
3533									0.8925	0.9962	0.8167	0.5984	0.7889	
3534									1.1181	1.1138	0.9986	1.0012	1.3369	
3535									0.8917	0.8380	0.9138	0.7879	0.9331	
3536									0.9384	0.6051	0.5127	0.7545	0.8821	
3537									1.1894	1.0687	0.9488	1.8105	0.9597	
3538									0.2672	0.3000	0.2476	0.2487	1.3267	
3539									0.9992	0.9030	1.0700	1.2163	1.2885	
3540									1.0652	1.1069	1.5538	1.8774	0.8442	
3552	A_32_P38637	84626	NM_032534	KRBA1	KRAB-A domain containing									
3553	A_23_P35564	25956	NM_015490	SEC31B	SEC31 homolog B (S. cere									
3554	A_24_P811397		_	A_24_P811397										
3555	A_23_P80827	84248	NM_001011537	FYTTD1	forty-two-three domain con									
3556	A_23_P88849	6236	NM_001128850	RRAD	Ras-related associated wit									
3557	A_23_P356965		_	A_23_P356965										
3558	A_23_P106505	9836	NM_014793	LCMT2	leucine carboxyl methyltran									
3559	A_23_P139376	6188	NM_001005	RPS3	ribosomal protein S3									
3560	A_32_P135348	85461	NM_033394	TANC1	tetratricopeptide repeat, an									
3561	A_24_P256325	51455	NM_001037872	REV1	REV1 homolog (S. cerevisi									
3562	A_32_P57465	2356	NM_001018078	FPGS	folylpolyglutamate synthas									
3563	A_23_P64313	26242	_	OR4C1P	olfactory receptor, family 4									
3564	A_23_P145485	80328	NM_025217	ULBP2	UL16 binding protein 2									
3565	A_23_P327451	4883	NM_000908	NPR3	natriuretic peptide receptor									

B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10
E3521
estrogen receptor 1
E2-mediated Regulation of ESR1 mRNA Expression
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
Log2 Relative Ratio of mRNA Levels of 10-9M E2/CON-treated cells
0
1
2
3
4
5
6
7
8
9
Time (days)
MCF7/WS8
MCF7/5C
MCF7/2A (S)
MCF7/2A (L)
E2-mediated Regulation of ESR1 mRNA Expression
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
Log2 Relative Ratio of mRNA Levels of 10-9M E2/CON-treated cells
0
24
48
72
96
Time (h)
MCF7/WS8
MCF7/5C
MCF7/2A
log-profiles
Ready
NUM

1	ProbeName	EntrezID	Refseq	Symbol	GeneName	LR.5C.2	LR.5C.6	LR.5C.12	LR.5C.24	LR.5C.48	LR.5C
3521	A_23_P309739	2099	NM_000125	ESR1	estrogen receptor 1	-0.1743	-0.4187	-0.4338	-1.2402	-1.5466	
3522						790	0.6090	0.4468	0.2460	-0.1265	
3523						783	-0.0199	-0.0444	0.0399	0.1271	
3524						404	-0.0754	-0.1962	-0.3037	-0.2569	
3525						356	0.0749	0.1171	0.0816	-0.1444	
3526						825	-0.4311	-0.1636	-0.4645	-0.4747	
3527						421	0.1796	-0.1010	0.2269	0.0533	
3528						840	0.5500	0.1731	-0.3485	-0.4827	
3529						288	0.5116	0.2130	0.2504	0.1205	
3530						222	0.6408	0.8244	1.1073	0.9939	
3531						335	-0.1749	-0.0777	-0.1662	-0.2668	
3532						438	-0.1743	-0.1018	-0.2960	-0.5284	
3533						569	-0.4156	-0.0193	-0.1759	-0.0289	
3534						533	0.1494	-0.0562	0.1497	0.1549	
3535						690	-0.3782	-0.3193	-0.1810	-0.3139	
3536						452	0.0965	0.0219	-0.1143	-0.7306	
3537						146	0.2737	-0.0398	0.1905	0.0852	
3538						278	-0.5974	-1.3304	-1.9066	-1.7375	
3539						575	-0.0364	-0.0421	-0.0625	-0.1709	
3540						759	-0.2479	-0.1090	0.0298	0.1236	
3552	A_32_P38637	84626	NM_032534	KRBA1	KRAB-A domain con						
3553	A_23_P35564	25956	NM_015490	SEC31B	SEC31 homolog B (						
3554	A_24_P811397		_	A_24_P811397							
3555	A_23_P80827	84248	NM_001011537	FYTTD1	forty-two-three doma						
3556	A_23_P88849	6236	NM_001128850	RRAD	Ras-related associat						
3557	A_23_P356965		_	A_23_P356965							
3558	A_23_P106505	9836	NM_014793	LCMT2	leucine carboxyl met						
3559	A_23_P139376	6188	NM_001005	RPS3	ribosomal protein S3						
3560	A_32_P135348	85461	NM_033394	TANC1	tetratricopeptide rep						
3561	A_24_P256325	51455	NM_001037872	REV1	REV1 homolog (S. c						
3562	A_32_P57465	2356	NM_001018078	FPGS	folylpolyglutamate sy						
3563	A_23_P64313	26242	_	OR4C1P	olfactory receptor, f						
3564	A_23_P145485	80328	NM_025217	ULBP2	UL16 binding protein						
3565	A_23_P327451	4883	NM_000908	NPR3	natriuretic peptide re						

Figure 1. Screenshots of the cell line microarray database and automated graphs of E$_2$-downregulated ESR1 (ERα) mRNA expression. (A) Linear scaled plots. **(B)** Log$_2$ scaled plots. The upper left-hand inset graphs show all 4 time course profiles including the MCF-7:2A long-term time course, while the lower right-hand inset graphs show just the short 96 h time course profiles.

Task 4b. Identify regulatory networks for pathways indicative of differential responses to E_2.

WORK ACCOMPLISHED

Significant Differentially Expressed Genes

We sought to determine which genes were differentially regulated by E_2 in estrogen deprivation-resistant MCF-7:5C and MCF-7:2A cells compared to wild-type MCF-7:WS8 cells.

Probe intensity data were in the form of ratios of E_2/Control treatment due to the nature of the competitive hybridization process of using the Agilent 2-channel microarrays. Normalized signal ratio values were obtained from Agilent Human 4x44k arrays using Agilent's Feature Extraction (FE) software (v9.1), which incorporates a spatial detrending background correction method, loess transformation for intensity-dependent within-array dye normalization, and a surrogate value substitution of very low intensity values near background. Agilent probes were removed if they, according to Agilent FE software, (i) showed poor spot quality measures on more than one array; or (ii) had intensities near background such that their expression values were replaced by surrogate values in the FE software across both channels and all arrays. After filtering, 29,634 probes were used in the differential expression analysis.

As a starting point, single time points were examined. Probes were retained that showed at least a 2-fold difference in relative intensity ratios with a *P*-value ≤ 0.0001 between any pairwise combination of WS8, 5C and 2A cells. Since this analysis required comparing matched time points between cell lines, the MCF-7:2A 3-9 day time course data could not be considered. Differential expression was assessed using empirical Bayes moderated two-sample t-statistics as outlined in LIMMA (Linear Models for Microarray Data) (2). LIMMA borrows strength across genes and identifies genes with statistically significant changes in expression by combining information from a set of gene-specific tests. It is suitable for analyzing microarray data involving factorial designs (multiple conditions) and enables to extract relevant contrasts (treatment combinations) of interest for further analysis. The LIMMA module in the open source Bioconductor suite [www.bioconductor.org, (1)] was utilized. All computations were performed using the open source R statistical language and environment [www.r-project.org, (3)]. A total of 10,303 probes were identified as being at least 2-fold different with a *P*-value ≤ 0.001 in at least one time point between any pairwise combination of WS8, 5C and 2A cells.

Gene Set Enrichment and Gene Ontology Analyses

The significant differentially expressed genes were examined for enrichment of those that map to a particular curated category (pathway, process, etc.) using GSEA (Gene Set Enrichment Analysis) (4). GSEA involves rank ordering all significantly regulated genes in a list (L) and determining whether a set of genes in a given biologic process (S; as determined by prior knowledge) are randomly distributed across the entire list of rank ordered genes or whether they are primarily ranked together at the top or bottom of the list, and therefore co-regulated as a group of genes. By proceeding stepwise from the top to the bottom of the ranked gene list L, a running-sum statistic is increased each time a gene is found in a given biologic process S and decreasing it when the gene is not in S. An enrichment Score (ES) is reported that corresponds to the maximum deviation from zero of the running-sum statistic. Curated biological process gene sets S were obtained from KEGG, GenMapp and other pathway sources available from GSEA's MsigDB database. Gene sets that have more then 500 or less than 15 genes were excluded. The genes in the list L were preranked according to fold-differences between the paired cell lines, and then GSEA was applied using default settings (1000 permutations and weighted enrichment statistic). Gene sets that were identified at a P-value ≤ 0.05 and a false discovery rate (FDR) Q-value ≤ 0.25 were defined as significantly enriched. The results of the GSEA are shown in Tables 1 – 3.

The significant differentially expressed genes were also examined for over-representation of gene ontologies. Gene ontology (GO) is a unified categorization system of gene products based on annotation, which describes the gene product using defined terms corresponding to cellular components, molecular functions, and biological processes. Conditional hypergeometric test statistics as implemented by the GOstats tool in the Bioconductor suite were used to evaluate the over-representation of GO categories among differentially expressed genes. A *P*-value < 0.01 was used to select significantly over-represented GO categories.

Table 1. 5C vs. WS8 Significantly Different Co-regulated Gene Sets

5C-WS8 Cells Pairwise Comparison					
Time point	**Enriched Gene Set**	**Gene Expression**	**Genes in Set**	**P-value**	**FDR Q-value**
2 h	none				
6 h	none				
12 h	CYTOKINE - CYTOKINE RECEPTOR INTERACTION	Increased	11	0.002	0.013
	REGULATION OF ACTIN CYTOSKELETON	Increased	10	0.004	0.007
	BREAST CANCER - ESTROGEN SIGNALING	**Decreased**	**10**	**0.020**	**0.065**
24 h	**BREAST CANCER - ESTROGEN SIGNALING**	**Decreased**	**17**	**0.047**	**0.050**
48 h	REGULATION OF ACTIN CYTOSKELETON	Increased	28	0.031	0.17
	CYTOKINE - CYTOKINE RECEPTOR INTERACTION	Increased	18	0.047	0.12
	BREAST CANCER - ESTROGEN SIGNALING	**Decreased**	**20**	**0.004**	**0.027**
	PURINE METABOLISM	Decreased	15	0.042	0.092
	TGF BETA SIGNALING PATHWAY	Decreased	19	0.035	0.082
72 h	CYTOKINE - CYTOKINE RECEPTOR INTERACTION	Increased	35	0.006	0.12
	BREAST CANCER - ESTROGEN SIGNALING	**Decreased**	**28**	**0.054**	**0.23**
96 h	CYTOKINE - CYTOKINE RECEPTOR INTERACTION	Increased	50	< 0.0001	< 0.0001
	TOLL LIKE RECEPTOR SIGNALING PATHWAY	Increased	22	< 0.0001	0.003
	APOPTOSIS	**Increased**	**22**	**< 0.0001**	**0.013**
	SMOOTH MUSCLE CONTRACTION	Increased	39	0.011	0.12
	JAK-STAT SIGNALING PATHWAY	Increased	30	0.013	0.15
	HEMATOPOIETIC CELL LINEAGE	Increased	15	0.017	0.16
	B CELL RECEPTOR SIGNALING PATHWAY	Increased	15	0.022	0.14
	HIV NEF PATHWAY	Increased	15	0.023	0.13
	GNRH SIGNALING PATHWAY	Increased	19	0.030	0.15
	EPITHELIAL CELL SIGNALING IN HELICOBACTER PYLORI INFECTION	Increased	17	0.030	0.16
	WNT SIGNALING PATHWAY	Increased	41	0.039	0.17
	CELL ADHESION MOLECULES	Increased	22	0.045	0.16
	HEDGEHOG SIGNALING PATHWAY	Increased	16	0.051	0.16
	G1 to S PHASE CELL CYCLE REACTOME	**Decreased**	**18**	**0.002**	**0.080**
	PYRIMIDINE_METABOLISM	Decreased	16	0.009	0.16
	CELL CYCLE_KEGG	**Decreased**	**23**	**0.014**	**0.15**
	GLYCEROLIPID METABOLISM	Decreased	15	0.016	0.11
	BREAST CANCER - ESTROGEN SIGNALING	**Decreased**	**32**	**0.018**	**0.13**
	CELL COMMUNICATION	Decreased	24	0.039	0.13
	CELL CYCLE	**Decreased**	**34**	**0.043**	**0.18**
	PYRIMIDINE METABOLISM	Decreased	18	0.048	0.19

Table 2. 2A vs. WS8 Significantly Different Co-regulated Gene Sets

Time point	Enriched Gene Set	Gene Expression	Genes in Set	P-value	FDR Q-value
2 h	none				
6 h	none				
12 h	none				
24 h	none				
48 h	NEUROACTIVE LIGAND RECEPTOR INTERACTION	Decreased	24	< 0.0001	0.072
72 h	METABOLISM OF XENOBIOTICS BY CYTOCHROME P450	Increased	18	< 0.0001	0.032
	CELL COMMUNICATION	Increased	31	0.019	0.067
	CELL ADHESION MOLECULES	Decreased	23	0.005	0.25
96 h	CELL COMMUNICATION	Increased	30	0.008	0.13

Table 3. 5C vs. 2A Significantly Different Co-regulated Gene Set

Time point	Enriched Gene Set	Gene Expression	Genes in Set	P-value	FDR Q-value
2 h	none				
6 h	none				
12 h	none				
24 h	CELL ADHESION MOLECULES	Increased	18	0.019	0.24
	BREAST CANCER - ESTROGEN SIGNALING	**Decreased**	**22**	**0.078**	**0.16**
48 h	CELL COMMUNICATION	Decreased	28	< 0.0001	0.004
72 h	GLYCAN STRUCTURES BIOSYNTHESIS_2	Increased	16	0.014	0.21
	CELL COMMUNICATION	Decreased	30	< 0.0001	0.004
	METABOLISM OF XENOBIOTICS BY CYTOCHROME P450	Decreased	16	0.013	0.041
	BREAST CANCER - ESTROGEN SIGNALING	**Decreased**	**33**	**0.024**	**0.095**
96 h	T CELL RECEPTOR SIGNALING PATHWAY	Increased	16	0.018	0.24
	CELL COMMUNICATION	Decreased	31	< 0.0001	0.001

GSEA identified breast cancer-estrogen signaling gene sets in 5C cells compared to WS8 at 5 time points (Table 1) and in 5C cells compared to 2A cells at 2 time points (Table 3). It was expected to find this class of genes given the nature of the cell lines models. The core-enriched breast cancer-estrogen signaling genes are shown in a heat map in Fig. 2.

GSEA also identified a set of genes involved in apoptosis that were up-regulated in the 5C cells compared to WS8 cells at 96 h of E_2 treatment; these genes are shown in Fig. 3. In the apoptosis gene set identified in 5C cells at the 96 h E_2 treatment-time point, multiple genes in several important gene family were up-regulated including caspases (CASP4, CASP8, CASP10, and CASP1), pro-apoptotic Bcl2 family members [Bim (BCL2L11) and Bax], and tumor necrosis factor members (TNF and TNFRSF21). Inspection of the heat map in Fig. 3 indicated that CASP4 was the first of the caspases to be induced, and that Bim was induced before Bax. Interestingly, both CASP4 (5) and the pro-apoptotic Bim (6) have been shown to be involved in endoplasmic reticulum stress-induced apoptosis. Furthermore, the gene ontology analysis indicated that the category cellular response to stress (GO:0033554) was over-represented in the 5C cells at 96 h of E_2 treatment ($P < 0.0001$). Therefore, further analyses focused on CASP4 and stress response genes as described under Task 2b-2. These studies under Task 2b-2 concluded that the estrogen-deprivation-resistant 5C and 2A cells displayed gene expression profiles indicative of unfolded protein stress, and that functional inhibition of CASP4 completely reversed E_2-inhibited growth of 5C cells.

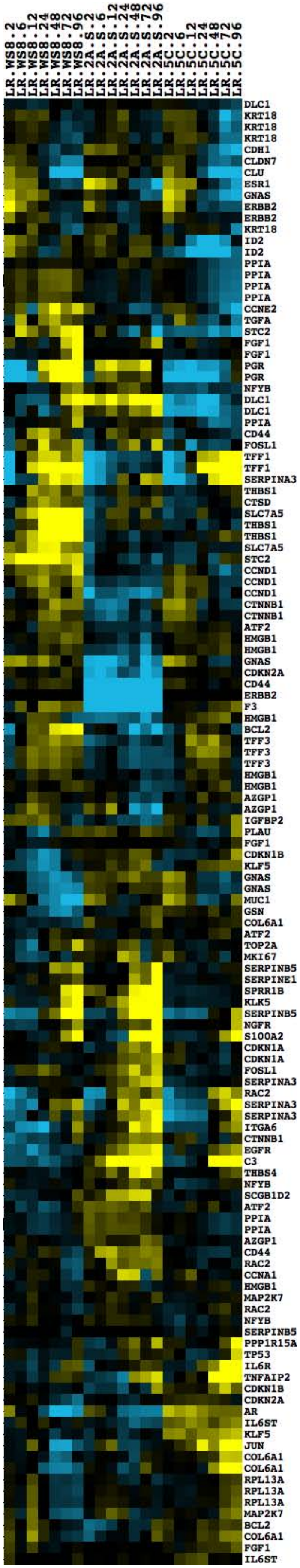

Figure 2. Core-enriched breast cancer - estrogen signaling genes identified by GSEA.

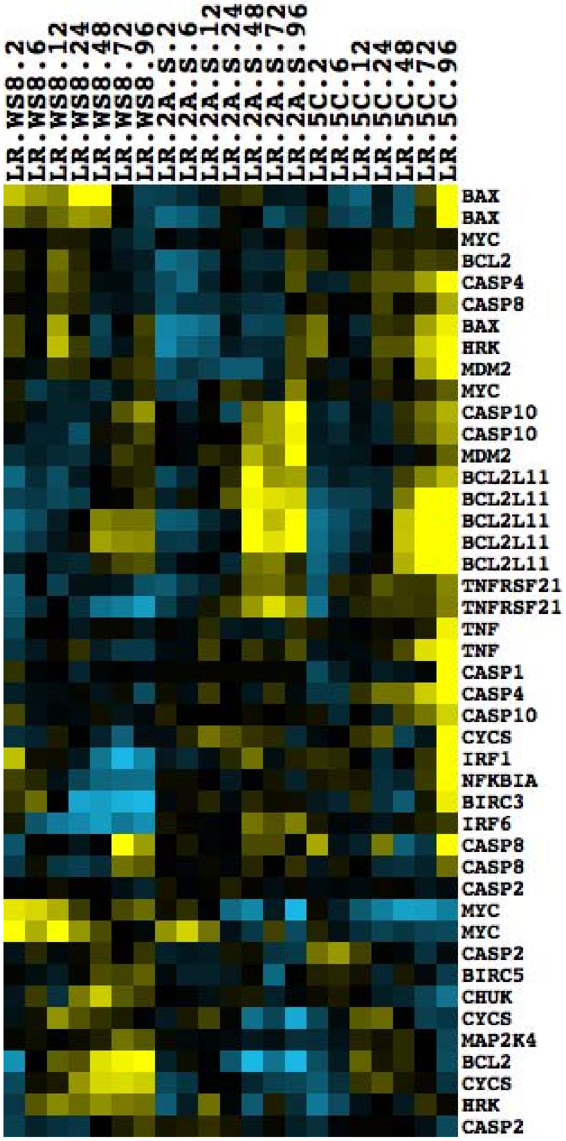

Figure 3. Apoptosis genes identified by GSEA.

KEY FINDINGS

- An Excel workbook database containing the summary information of the gene expression profiles was updated with the 2A microarray data. Visual Basic macros that produce 'on-the-fly' automated plots of expression profile of a selected gene were also updated and improved.

- Significant differentially expressed genes at single time points between all pairs of the 3 cell lines showing at least a 2-fold difference in expression were identified.

- The differentially expressed genes were analyzed for enriched pathways using GSEA and gene ontology terms. Breast cancer – estrogen signaling gene sets were identified at multiple time points. An apoptosis gene set was also identified in the 5C cells at the 96 h time point.

    - In the apoptosis gene set, caspases, pro-apoptotic Bcl2 members, and tumor necrosis factor members were found.
    - CASP4 was the first of the caspases to be E_2-induced, and pro-apoptotic Bim was induced before Bax.
    - Both CASP4 and Bim have been associated with endoplasmic reticulum stress-induced apoptosis.
    - Gene ontology analysis indicated that the category cellular response to stress was over-represented.

- Based on these results, gene expression profiles related to cellular response to stress and a functional role for CASP4 in E_2-induced apoptosis were evaluated as described under Task 2b-2.

Future Directions

These bioinformatic analyses focused on single time points. However, the expression data are profiles covering a time course. We are currently developing methods to define differential expression based on the entire time course profile rather than single time points.

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KEY RESEARCH ACCOMPLISHMENTS
(For all Tasks of the CoE)

Administration

- Dr. Jordan has accepted a position at GU as the Scientific Director and Vice Chairman of the Department of Oncology at the Lombardi Comprehensive Cancer Center. As a result, the primary site of the CoE has moved to GU. Fox Chase Cancer Center has written the letter of relinquishment, and the Lombardi Cancer Center is in the process of submitting the requirements for the transfer of the grant.

Task 1 (FCCC/Goldstein, Swaby)

- The clinical trial to evaluate dose de-escalation of estrogen (Estrace) to reverse antihormone resistance in patients treated exhaustively with antihormone therapy has enrolled two patients.
- Patient eligibility criteria has been amended to increase patient enrollment.
- Georgetown University and its clinical trial network, MedStar, have replaced Johns Hopkins University as an academic partner. Regulatory negotiations and approval to open the clinical trial at Georgetown University are currently underway.
- Two Fox Chase Partner network sites will be participating in the clinical trial

Task 2a (FCCC/Jordan, Ariazi)

- Generated 3 complete sets of protein lysates for proteomic analyses, with each set including MCF-7:WS8, MCF-7:5C, and MCF-7:2A cells.

Task 2b-1 (FCCC/Jordan, Ariazi)

- GPR30 was overexpressed and exhibited parallel increases in functional activity in estrogen deprivation-resistant MCF-7/5C and MCF-7/2A cells compared to wild-type MCF-7 cells.
- Mining of the breast carcinoma microarray data representing 1,250 specimens across 5 independent cohort showed increased GPR30 expression associated with ERα–positive status.
- E_2 decreased both ERα and GPR30 mRNA levels, but the GPR30 specific agonist G-1 did not, indicating that E_2's effect on ERα and GPR30 expression was mediated by ER.
- RNA interference-mediated depletion of GPR30 blocked E_2– and G-1–induced Ca^{2+} mobilization, but ERα depletion did not. Instead, ERα knockdown augmented the E_2-induced Ca^{2+} response; likely due to ERα depletion leading to increased GPR30 expression.
- In proliferation studies, GPR30 knockdown promoted, whereas G-1 profoundly inhibited E_2-stimulated growth of MCF-7 cells. Consistent with increased growth in GPR30-depleted cells, CXCR4 and E_2-induced SDF1α expression were increased, while in G-1–treated and growth inhibited cells, E_2-induced SDF1α expression was reduced but CXCR4 expression was unaffected.
- Flow cytometry showed that G-1 prevented E_2-stimulated cells from entering S phase. Concurrently, p53, p21, and G(1)-phase specific cyclin D1 accumulated, while the G(2)/M-phase specific cyclin B1 did not accumulate further supporting a G(1)-phase cell cycle block.

Task 2b-2 (FCCC/Jordan, Ariazi)

- E_2 induced CASP4, CASP5, CASP10, CASP1, and CASP8 in resistant 5C cells. CASP4 and CASP5 were the first of the caspases to be induced, and these were the only caspases induced in resistant 2A cells (Fig. 3).
- Gene ontology analysis showed deregulated stress response factors were over-represented in the resistant cells. (Fig. 4).
- Examination of stress response gene expression profiles showed a general pattern of induction by E_2 selectively in wild-type MCF-7:WS8 cells, and significantly decreased induction in estrogen-deprivation resistant MCF-7:5C and MCF-7:2A cells (Figs. 5-9).
- The stress response genes indicated that E_2 led to endoplasmic reticulum stress (Fig. 5) and induction of pro-apoptotic Bim (Fig. 6).
- Not only was endoplamic reticulum stress indicated by the expression profiles, but also an overall deficiency in protein folding (Figs. 7-9).
- The endoplasmic reticulum stress-induced CASP4 was functionally required in E_2-induced apoptosis.
  - E_2-induced CASP4 mRNA (Fig. 10A) and protein levels in 5C cells.
  - CASP4 was cleaved in E_2-treated 5C cells.
  - Inhibition of CASP4 using Z-LEVD-FMK completely blocked E_2-induced PARP cleavage (Fig. 10B), reversed E_2-inhibited growth, and prevented E_2-induced apoptotic morphologic alterations in 5C cells.

Task 2b-3 (FCCC/Jordan, Fan)

- Estrogen deprivation-resistant MCF-7:5C cells exhibited high levels of activated c-Src, leading to greater sensitivity to the c-Src inhibitor PP2 compared to wild-type MCF-7:WS8 cells. This demonstrates that the c-Src tyrosine kinase was involved in adaptation of MCF-7 cells to estrogen deprivation.
- Quite surprisingly, selection of MCF-7:5C cells under E_2 plus PP2 conditions produced a cell line in which E_2 did not induce apoptosis, and instead dramatically stimulated growth.
- Selection of MCF-7:5C cells under E_2 plus PP2 conditions also allowed SERMs to manifest greater agonist activity by promoting growth.

Task 2b-4 (FCCC/Jordan, Sengupta)

- XBP1 depletion dramatically inhibited E_2-stimulated growth of ERα-positive breast and endometrial cancer cells.
- Depletion of XBP1 down-regulated the expression of Bcl-2 levels in MCF-7 and ECC1 cells.
- Depletion of Bcl-2 levels also dramatically inhibited E_2-stimulated growth of ERα-breast and endometrial cancer cells, suggesting that the growth inhibitory effects of XBP1 depletion may have been mediated at least partly through decreased Bcl-2 levels.
- Over-expression of XBP1 in MCF-7 cells modestly increased Bcl-2 levels selectively in the presence of E_2.
- XBP1 physically interacted with ERα protein in MCF-7 cells in a ligand-independent manner.

- XBP1 levels (depletion or over-expression) do not modulate classical transcriptional activity of ERα mediated through binding of an ERE.
- MCF-7 cells stably transfected with XBP1 are significantly more resistant to paclitaxel (taxol) induced toxicity, suggesting higher expression of XBP1 can potentially reduce the therapeutic efficacy of chemotherapeutic agents.

Task 3 (GU/Riegel and Wellstein)

- We have completed our proteomics analysis to identify AIB1 and pY-complex proteins that are regulated differentially in response to E_2 in MCF-7 versus MCF-7/5C cells.
- We have built a pathway model that integrates this information into the differential response of these cells to E_2 (growth vs. apoptosis) and can now interrogate the relative contribution of these pathways revealed by the initial analysis.
- We have built an AIB1 interaction network that allows us to find functional association with pathways that have druggable targets.
- We have established MS analysis of the ER and found differential posttranslational modifications in the ER when comparing MCF-7 and MCF-7/5C cells. This should reveal additional signaling mechanisms that impact on ER activity and will be connected to the findings already in place.

Task 4a-4c (TGen/Cunliffe)

- Completed gene expression profiling on MCF-7:2A cell line induced with estrogen over a short (96h) and long (9 day) time course. Data provided back to the CoE for joint analysis.
- Developed inflection-based analysis of genes expression profiles to identify genes that rapidly change in response to E_2. This analysis was applied to MCF-7:WS8, MCF-7:5C and MCF-7:2A (long-term time course) cells.
- Developed and applied differential network analytical methodology as a novel approach to characterizing pathways related to E_2-induced apoptosis in the MCF-7:5C cell line model.
- Completed high throughput RNAi screen of the resistant MCF-7:5C and wild-type MCF-7:WS8 cells. Successfully identified a small number of novel gene ‘hits’ that are protective against E_2-induced apoptosis. These candidates are being validated in “flexiplate” format in the Azorsa Laboratory.

Task 4a-4b (FCCC/Jordan, Ariazi)

- An Excel workbook database containing the summary information of the gene expression profiles was updated with the 2A microarray data. Visual Basic macros that produce ‘on-the-fly’ automated plots of expression profile of a selected gene were also updated and improved.
- Significant differentially expressed genes at single time points between all pairs of the 3 cell lines showing at least a 2-fold difference in expression were identified.
- The differentially expressed genes were analyzed for enriched pathways using GSEA and gene ontology terms. Breast cancer – estrogen signaling gene sets were identified at multiple time points. An apoptosis gene set was also identified in the 5C cells at the 96 h time point.
    - In the apoptosis gene set, caspases, pro-apoptotic Bcl2 members, and tumor necrosis factor members were found.
    - CASP4 was the first of the caspases to be E_2-induced, and pro-apoptotic Bim was induced before Bax.

  - Both CASP4 and Bim have been associated with endoplasmic reticulum stress-induced apoptosis.
  - Gene ontology analysis indicated that the category cellular response to stress was over-represented.
- Based on these results, gene expression profiles related to cellular response to stress and a functional role for CASP4 in E_2-induced apoptosis were evaluated as described under Task 2b-2.

REPORTABLE OUTCOMES

Publications

1. Oseni TS, Patel RP, Pyle JR, Jordan VC. Selective estrogen receptor modulators and phytoestrogens. *Planta Medica* 2008: 74(13):1656-65.

2. Oh AS, Lahusen JT, Chien CD, Fereshteh MP, Zhang X, Dakshanamurthy S, Xu J, Kagan BL, Wellstein A, Riegel AT. Tyrosine phosphorylation of the nuclear receptor coactivator AIB1/SRC-3 is enhanced by Abl kinase and is required for its activity in cancer cells. *Mol Cell Biol.* 2008: 28(21):6580-93.

3. Lewis-Wambi JS, Kim HR, Wambi C, Patel R, Pyle J, Klein-Szanto AJ, Jordan VC. Buthionine sulfoximine sensitizes hormone-resistant human breast cancer cells to estrogen-induced apoptosis. *Breast Cancer Res* 2008: 10(6):R104.

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5. Jordan VC. A century of deciphering the control mechanisms of estrogen action in breast cancer: the origins of targeted therapy and chemoprevention. *Cancer Res* 2009: 69(4):1243-1254.

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12. Lewis-Wambi JS, Jordan VC. Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? *Breast Cancer Research* 2009: 11(3):206.

13. Lahusen T, Henke R, Kagan B, Wellstein A, Riegel A. The role and regulation of the nuclear receptor co-activator AIB1 in breast cancer. *Breast Cancer Research and Treatment* 2009: 116(2):225-237.

14. Wambi C, Sanzari J, Sayers CM, Nuth M, Zhou Z, Davis J, Finnberg N, Lewis-Wambi JS, Ware JH, El-Deiry WS, Kennedy AR. Effect of dietary antioxidants on proton total body irradiation mediated hematopoietic cell and animal survival. *Radiation Research* 2009: 172:175-186.

15. Brauch H, Jordan VC. Targeting of tamoxifen to enhance antitumour action for the treatment and prevention of breast cancer: The “personalised” approach? *European Journal of Cancer* 2009: 45 (13): 2274-2283.

16. Jordan VC, Lewis-Wambi JS, Patel R, Kim H, Ariazi EA. New Hypotheses and Opportunities in Endocrine Therapy: Amplification of Oestrogen-Induced Apoptosis. *Breast* 2009 (in press).

17. Morrow M, Chatterton Jr. RT, Rademaker AW, Hou N, Jordan C, Hendrick E, Khan SA. A Prospective Study of Variability in Mammographic Density During the Menstrual Cycle. *Breast Cancer Research and Treatment* 2009 (in press).

18. Patel RR, Sengupta S, Kim HR, Klein-Szanto A, Pyle JR, Zhu F, Ross EA, Fargnoli J, Jordan VC. Experimental Treatment of Estrogen Receptor (ER) Positive Breast Cancer with Tamoxifen and Brivanib Alaninate, a VEGFR2/FGFR1 Kinase Inhibitor: a novel clinical application of angiogenesis inhibitors. *European Journal of Cancer* 2009 (submitted).

19. Ariazi EA, Yerrum S, Brailoiu E, Shupp HA, Slifker MJ, Cunliffe HE, Black MA, Donato AL, Arterburn JB, Oprea TI, Prossnitz ER, Dun NJ, Jordan VC. The G Protein-Coupled Receptor GPR30 Inhibits Proliferation of Estrogen Receptor-Positive Breast Cancer Cells. *Cancer Research* 2009 (submitted).

20. Peng J, and Jordan VC. Expression of estrogen receptor alpha with a Tet-off adenoviral system induces G0/G1 cell cycle arrest in SKBr3 breast cancer cells. *International Journal of Oncology* 2009 (submitted)

21. Hu Z, Kagan BL, Ariazi E, Zhang L, Huang H, Wu C, Riegel AT, Jordan VC, Wellstein A. Distinct pathways control estradiol-induced growth and apoptosis in breast cancer cells. *Proceedings of the National Academy of Sciences* 2009 (in preparation).

Abstracts

1. *Abstract #2502 was published in the 2009 Proceedings of the 100th Annual Meeting of the American Association for Cancer Research, Denver, CO, April 18-22, 2009.*
   Cross-talk between GPR30 and estrogen receptor alpha (ERα): ERα mediates estrogen-induced down-regulation of GPR30 expression and the GPR30 agonist G-1 induces ERα-Ser118 phosphorylation.
   Eric A. Ariazi, Smitha Yerrum, Surojeet Sengupta, Heather A. Shupp, Anne L. Donato, Heather E. Cunliffe, V. Craig Jordan.

2. *Abstract #2338 was published in the 2009 Proceedings of the 100th Annual Meeting of the American Association for Cancer Research, Denver, CO, April 18-22, 2009.*
   17β-Estradiol Impairs the Growth of Selective Estrogen Receptor Modulator (SERM) resistant endometrial tumors.
   Gregor M. Balaburski, Rita Dardes, V. Craig Jordan.

3. *Abstract #4606 was published in the 2009 Proceedings of the 100th Annual Meeting of the American Association for Cancer Research, Denver, CO, April 18-22, 2009.*
   Bazedoxifene (TSE-424) is a potent inhibitor of long-term estrogen deprived breast cancer cells.
   Joan S. Lewis-Wambi, Helen R. Kim, V. Craig Jordan

4. *Abstract #3285 was published in the 2009 Proceedings of the 100th Annual Meeting of the American Association for Cancer Research, Denver, CO, April 18-22, 2009.*
   Pathway and network analysis of E2-induced apoptosis in breast cancer cells.
   Zhang-Zhi Hu, Benjamin Kagan, Hongzhan Huang, Hongfang Liu, V. Craig Jordan, Anna T. Riegel, Anton Wellstein, Cathy Wu.

5. *Abstract was presented during the IMPAKT Breast Cancer Conference, European Society for Medical Oncology, Brussels, Belgium, May 7-9, 2009 and published in Annals of Oncology* 2009: 20(2):11-12.
   Back to Basics: Oestrogen To Kill Anti-Hormone Resistant Breast Cancer.
   V. Craig Jordan, Joan Lewis-Wambi, Helen Kim, Ping Fan, Eric Ariazi.

6. *Abstract was presented at the TGEN Annual Scientific Retreat, June 4, 2009.*
   Genomic Evolution of Endocrine-Resistant Breast Cancer Cell Lines Reveals Molecular Aberrations Consistent with Biological Phenotype.
   Catherine M. Mancini, Pilar Ramos, Amanda L. Willis, Megan L. Russell, Ping Fan, Joan S. Lewis-Wambi, Eric A. Ariazi, Helen R. Kim, Coya Tapia, Michael Bittner, V. Craig Jordan, Heather E. Cunliffe.

7. *Abstract was presented at the TGEN Annual Scientific Retreat, June 4, 2009.*
   A New Therapeutic Paradigm for Breast Cancer: Exploiting Low-Dose Estrogen-Induced Apoptosis.
   Pilar Ramos, Eric A. Ariazi, Amanda L. Willis, Yoganand Balagurunathan, David Azorsa, Meredith Henderson, Seungchan Kim, Michael Bittner, V. Craig Jordan, Heather E. Cunliffe.

8. *Abstract #P3-72 was presented during the Endocrine Society Annual Meeting, Washington, D.C., June 10-13, 2009.*

The roles of AIB/SRC-3 and erbB-2/HER2 in the estrogen-induced apoptosis of human breast cancer cells.
Benjamin L. Kagan, Zhang-Zhi Hu, V. Craig Jordan, Anna T. Riegel, Anton Wellstein.

Presentations

2008

1. Jordan, VC. Estrogen in the life and death of breast cancer cells, Loyola University Medical Center, Cardinal Bernardin Cancer Center, October 9, 2008.
2. Jordan, VC. Estrogen in The Life and Death of Breast Cancer Cells, 10th Annual Lynn Sage Memorial Lecture, 10th Annual Lynn Sage Breast Cancer Symposium, October 24, 2009.
3. Jordan, VC. Estrogen in the life and death of breast cancer cells, Division of Cancer Medicine, Grand Rounds, M.D. Anderson Cancer Center, Houston, TX, November 4, 2008.
4. Cunliffe, H. A New Therapeutic Paradigm for Breast Cancer, Exploiting Low-Dose Estrogen-Induced Apoptosis, Breast Cancer Genetics and Genomics Symposium, Auckland Medical School, New Zealand, November 18, 2008.
5. Jordan, VC. Evolving Hormonal Strategies of the Treatment and Prevention of Breast Cancer, 12th Annual Cancer Congress, Moscow, Russia, November 19, 2008.
6. Jordan, VC. Opportunities to Reverse Antihormonal Drug Resistance and Advance Molecular Medicine, Madison, Wisconsin, November 2008.
7. Jordan, VC. Cancers vs. DES & Other (Anti-)Oestrogens (Cellular Aspects), The State of the Art on DES in 2008, UCB Pharma, Paris, France, December 8, 2008.
8. Jordan, VC. Cancers vs. DES & Other Anti-Oestrogens: Clinical Aspects, The State of the Art on DES in 2008, UCB Pharma, Paris, France, December 8, 2008.

2009

1. Jordan, VC. Are there personalized strategies for overcoming resistance to endocrine therapy?, 2nd Annual Symposium on Personalized Therapies for Breast Cancer, Miami, FL, January 25, 2009.
2. Jordan, VC. Defeating Drug Resistance to SERMs: Building on the Success of Tamoxifen and Raloxifene, Dr. Margarete Fischer-Bosch-Institut für Klinische Pharmakologie, Stuttgart, Germany, February 5, 2009.
3. Jordan, VC. New Hypotheses and Opportunities in Endocrine Therapy: Amplification of Oestrogen-Induced Apoptosis, Primary Therapy of Early Breast Cancer 11th International Conference, St. Gallen, Switzerland, March 11, 2009.
4. Jordan, VC. Oestrogen Action and the Life or Death of Breast Cancer, Royal Society of Medicine, Jephcott Lecture, London, England, April 7, 2009.
5. Cunliffe, H. A New Therapeutic Paradigm for Breast Cancer: Exploiting Low-Dose Estrogen-Induced Apoptosis, TGEN Annual Cancer and Cell Biology Division Retreat, Civic Center, Phoenix, AZ, April 25, 2009.
6. Jordan, VC. The Consequence of Exhaustive Antihormone Therapy: Estrogen Kills Breast Cancer Cells, New York Metropolitan Breast Cancer Group, Glenn Robbins Award, New York, NY, April 28, 2009.
7. Jordan, VC. The Consequence of Exhaustive Antihormone Therapy: Estrogen Kills Breast Cancer Cells, Vincent T. Lombardi Comprehensive Cancer Center, Georgetown University, Grand Rounds, Washington, D.C., May 1, 2009.
8. Jordan, VC. Back to Basics: Oestrogen to Kill Anti-Hormone Resistant Breast Cancer: Build on That, IMPAKT Breast Cancer Conference, European Society for Medical Oncology, Brussels, Belgium, May 7, 2009.

9. Jordan, VC. Tamoxifen, Raloxifene, SERMs, and Beyond, University of Crete, Heraklion, Crete, Greece, May 14, 2009.
10. Jordan, VC. A Solution to the Clinical Problem of Antihormone Drug Resistance in Breast Cancer. 29th Annual Conference of the German Society of Senology, Dusseldorf, Germany, June 11, 2009.
11. Jordan, VC. Deciphering the Control Mechanisms of Estrogen Action in Breast Cancer, Keynote Forum: Scientific Innovations for Future Anticancer Medicine, 2nd World Cancer Congress, Gateway to Future Medicine, Beijing, China, June 22, 2009.
12. Jordan, VC. Evolving Understanding of Estrogen Action and its Application for Breast Cancer Treatment, Midwest Breast Cancer Symposium, University of Iowa, Iowa City, IA, July 17, 2009.
13. Jordan, VC. Improving Refractory Endocrine Therapy with Agents to Inhibit Angiogenesis, Bristol-Myers Squibb, Princeton, New Jersey, August 19, 2009.
14. Jordan, VC. Estrogen Action for Growth and Apoptosis in Breast Cancer, World Class University Symposium, Dankoo University, Seoul, South Korea, August 25-26, 2009.

Grants

Anna Riegel (Principal Investigator)
Anton Wellstein (Co-Principal Investigator)
Submitted: Pre-application to NIH Challenge Grants in Health and Science Research (RC1)
Application Title**:** Role of A1B1 in hormone refractory cancer.
Mechanism: [0D09-003]-Recovery Act Limited Competition: NIH Challenge Grants in Health and Science Research (RC1)
Pre-application Submission Date: 05/08/09

Full-application Submission Date: The application was reviewed and has scored in the 10th percentile, and JIT has been submitted as of 07/27/09

Awards and Honorary Memberships

V. Craig Jordan
Jephcott Lecture and Medal from the Royal Society of Medicine (UK), 2009
Honorary Doctor of Medicine Degree, University of Crete, 2009
Fellow of the Academy of Medical Sciences (UK), 2009
Fellow of the Institute of Biology (UK), 2009
Member of the National Academy of Sciences, 2009

Appointments

Eric A. Ariazi, Ph.D.
July 1, 2009, Scientific Consultant to Dr. V. Craig Jordan and the Center of Excellence - BC050277 “A New Therapeutic Paradigm for Breast Cancer Exploiting Low-Dose Estrogen-Induced Apoptosis”.

Ping Fan, Ph.D.
July 1, 2009, Research Assistant Professor

V. Craig Jordan, O.B.E., Ph.D., D.Sc., FMedSci
July 1, 2009, Scientific Director at the Lombardi Comprehensive Cancer Center, Vice Chair of Department of Oncology, Professor of Oncology and Pharmacology

Helen Kim, B.S.
July 1, 2009, Laboratory Manager

Joan Lewis-Wambi, Ph.D.
July 1, 2009, Research Assistant Professor

Surojeet Sengupta, Ph.D.
July 1, 2009, Research Assistant Professor

CONCLUSION

Our consortium has made significant progress in interrogating E_2 regulated pathways involved in growth stimulation and apoptosis in breast cancer cells using unique long-term estrogen deprivation-resistant models developed in our laboratory.

We have collected all RNA samples **(Task 2a: FCCC/Ariazi and Jordan)**, and completed all the gene expression microarray hybridizations of the cell line models plus/minus E_2 **(Task 4a: TGen/Cunliffe)**; the estrogen-responsive MCF-7:WS8 and estrogen deprivation-resistant MCF-7:5C and MCF-7:2A cells microarrays were carried out covering 2 -96 h, and an additional 3 – 9 d time course of the MCF-7:2A cells to capture the time frame in which these cells undergo apoptosis. We catalogued all the summary values of the microarray data into a single Excel workbook and written Visual Basic macros that automatically produces plots of any selected gene across all the cell lines and time courses **(Task 4a: FCCC/Ariazi and Jordan)**. We have developed custom methods for the bioinformatic analyses of the microarray data. In prior years, we developed 3 methods for bioinformatic analysis including a template-based, distance-based and inflection-based method, and applied these methods to the MCF-7:WS8 and MCF-7:5C microarray data **(Year 2 report, Task 4b)**. In this current year, we applied the inflection-based method which finds genes showing rapidly changing expression, to the MCF-7:2A long-term 3 – 9 day time course array data **(Task 4b: TGen/Balagurnuathan and Cunliffe)**. This year, we also developed a genetic regulatory network (GRN) analysis method in which regulatory networks of genes are ‘learned’ based on genes that exhibit maximal changes in expression, and applied this GRN method to the MCF-7:WS8 and MCF-7:5C cells **(Task 4b: TGen/Kim and Cunliffe)**.

Additional methods of gene expression microarray data, such as Gene Set Enrichment Analysis (GSEA) and tests for over-representation of gene ontology terms, were also employed. GSEA indicated that a set of apoptosis genes were induced and gene ontology analysis indicated stress response genes were over-represented in estrogen deprivation-resistant cells **(Task 4b: FCCC/Ariazi and Jordan)**. In the set of identified apoptotic genes, multiple caspases were found, and of the caspases, caspase-4 was the first in the E_2 treatment time course to be induced in the MCF-7:5C cells, and was one of only two induced caspases in MCF-7:2A cells. Caspase-4 has been associated with endoplasmic reticulum stress, since gene ontology analysis indicated over-representation of stress response genes, the gene expression microarray data base was interrogated for genes involved in protein folding. Many chaperones and other factors involved in unfolded protein stress were found to be selectively induced by E_2 in the wild-type MCF-7:WS8 cells but lacked induction in both the resistant MCF-7:5C and MCF-7:2A cells, indicating an overall deficiency in protein folding in these resistant cells that would lead to accumulation and aggregation of unfolded proteins and eventually apoptosis. Furthermore, we verified that caspase-4 was induced by E_2 in both resistant cell lines but not in wild-type MCF-7:WS8 cells, and showed that inhibition of caspase-4 using Z-LEVD-FMK blocked E_2-induced PARP cleavage, E2-inhibited growth, and E2-induced morphologic changes consistent with apoptosis in the MCF-7:5C cells **(Task 2b-2: FCCC/Ariazi and Jordan)**.

Proteomic analyses have been carried out that identified proteins which differentially co-immunoprecipitated with the co-activator AIB1 or phospho-tyrosine complexes in an E_2-dependent manner in wild-type MCF-7:WS8 and resistant MCF-7:5C cells **(Task 3: GU/Riegel and Wellstein)**. Based on these proteomic results, an integrated pathway model has been built that reveals differential responses to E_2 involving growth versus apoptosis. Based on this network, the relative contribution of key factors can now be tested. We have also initiated a series of proteomic studies to identify differential post-translational modifications of immunoprecipitated estrogen receptor alpha (ERα) by liquid chromatography – mass spectrometry analysis. So far, we found 34 phosphorylation sites in ERα including some already well known. These phosphorylation sites will be related to E_2-induced growth versus apoptosis.

The high-throughput siRNA screening of the cell lines has proven technically challenging, but siRNA transfection conditions and cell viability assays have now been optimized. With optimized conditions in hand, the high-throughput siRNA screen of the MCF-7:WS8 and resistant MCF-7:5C cells have been carried out and several novel gene ‘hits’ that are protective of E_2-induced apoptosis are now being validated **(Task 4c: TGen/Azorsa and Cunliffe)**.

We have pursued mechanistic studies involving E_2 regulated pathways involving growth stimulation and apoptosis. Based on gene array expression data, caspase-4 was found and its inhibition blocked E_2-induced apoptosis as mentioned above. Microarray data has also shown that expression levels of the novel E_2-binding G protein-coupled receptor were increased in both the resistant MCF-7:5C and MCF-7:2A cells compared to wild-type MCF-7:WS8 cells, and since GPR30 binds ER ligands and mediates rapid non-genomic E_2 signaling events, GPR30 was investigated **(Task 2b-1: FCCC/Ariazi and Jordan)**. By mining publicly available gene expression microarray datasets, high GPR30 levels were found to associate with ERα-positive status in 5 independent cohorts covering 1,250 breast carcinomas. Therefore, GPR30 shared a statistical relationship with ERα, and functional studies were begun, but these have initially focused on the wild-type MCF-7:WS8 cells. We found that siRNA-mediated depletion of GPR30 promoted growth, while GPR30 pharmacological activation using the selective agonist G-1 dramatically blocked growth of wild-type MCF-7:WS8 cells. Additional experimentation showed that G-1 induced large increases in intracellular calcium concentrations, which likely led to the observed block at the G(1) phase in the cell cycle co-incident with induction of cell cycle inhibitors p53 and p21. Continuing studies involving GPR30 will focus on the resistant cell lines.

Also in the gene expression microarray data, the transcription factor XBP1 and the anti-apoptotic factor Bcl-2 were induced by E_2 to a greater extent in the wild-type MCF-7:WS8 cells compared to the resistant cells, and XBP1 and Bcl-2 are involved in endoplasmic reticulum stress, although Bcl-2 is involved in many pro-survival pathways. Therefore these factors were investigated and related to growth stimulation in the wild-type MCF-7:WS8 cells **(Task 2b-4: FCCC/Sengupta and Jordan)**. Depletion of XBP1 by siRNA-mediated methodology significantly inhibited E_2-stimulated growth of MCF-7:WS8 cells, and dramatically reduced Bcl-2 expression indicating that XBP-1 regulated Bcl-2 expression. XBP1-dependent regulation of Bcl-2 expression was further explored by showing XBP1 was recruited to the Bcl-2 promoter by chromatin immunoprecipitation (in Year 2 report), and that XBP1 overexpression led to increased Bcl-2 levels. The importance of Bcl-2 in E_2-stimulated growth was demonstrated by siRNA-mediated depletion of Bcl-2, which severely blocked growth of MCF-7:WS8 cells. Importantly, cells stably over-expressing XBP1 were resistant to chemotherapeutic toxicity caused by paclitaxel, demonstrating XBP1 as a pro-survival factor.

In an effort to combine agents with E_2 to additively or synergistically increase apoptosis in antihormone-resistant cells, the c-Src inhibitor PP2 was evaluated **(Task 2b-3: FCCC/Fan and**

Jordan), since c-Src has been shown to be functionally critical in tamoxifen resistance. To our surprise, long-term (2 months) treatment of MCF-7:5C cells with PP2 plus E_2 selected for a polyclonal cell line that appeared morphologically healthy and was no longer sensitive to E_2-induced apoptosis. In fact, withdrawal of E_2 plus PP2 followed by E_2 treatment again actually stimulated growth of these selected MCF-7:5C cells. The E_2-stimulated growth of these E_2 plus PP2-selected cells was shown to be ER dependent since the pure antiestrogen ICI 182,780 blocked this effect. Interestingly, the tamoxifen active metabolite 4-hydroxytamoxifen displayed increased agonist activity in these E_2 plus PP2-selected cells as demonstrated by growth stimulation. These E_2 plus PP2-selected MCF-7:5C cells will continue to be investigated using gene expression microarrays compared to the parental MCF-7:5C cells. Additionally, if we confirm that c-Src inhibitors reverse E_2-induced apoptosis in xenograft models, then these results may have important clinical implications for appropriately utilizing c-Src inhibitors in advanced antihormone-resistant breast cancers.

The clinical trial evaluating estrogen for the reversal of antihormone resistance in breast cancer **(Task 1: FCCC/Swaby and Goldstein)** has enrolled two patients and no dose limiting toxicities (DLTs) or serious adverse events (SAEs) have occurred. To increase enrollment, patient eligibility has been amended to remove barriers. John Hopkins University is no longer a planned clinical trial site; however, GU and its clinical trial network MedStar have replaced Johns Hopkins. Additionally, the trial will be opened in an additional FCCC partner site. It is anticipated that trial sites outside of FCCC will open enrollment by the first quarter of 2010.

Dr. Jordan has resigned from the Fox Chase Cancer Center (FCCC) and accepted a position as the Scientific Director of the Lombardi Comprehensive Cancer Center and Vice Chairman of the Department of Oncology at Georgetown University (GU). Hence the administration and the primary site of the CoE is in the process of being transitioned to GU **(Introduction)**. By the start of Year 3, the transition will have been completed. This move will streamline communications in the CoE as the proteomics site of the COE is already located at GU.

APPENDIX

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Selective Estrogen Receptor Modulators and Phytoestrogens

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Key words
- tamoxifen
- raloxifene
- phytoestrogens
- SERMs
- selective estrogen receptor modulators
- breast cancer
- chemoprevention

Abstract

Scientific achievements in the last two decades have revolutionized the treatment and prevention of breast cancer. This is mainly because of targeted therapies and a better understanding of the relationship between estrogen, its receptor, and breast cancer. One of these discoveries is the use of synthetic selective estrogen modulators (SERMs) such as tamoxifen in the treatment strategy for estrogen receptor (ER)-positive breast cancer. Hundreds of thousands of lives have been saved because of this advance. Not only is tamoxifen used in the treatment strategy for patients who have breast cancer, but also for prevention in high-risk premenopausal women. Another synthetic SERM, raloxifene, which was initially used to prevent osteoporosis, is also as effective as tamoxifen for prevention in high-risk postmenopausal women. In certain regions of the world, particularly in Asia, a low incidence of breast cancer has been observed. These women have diets that are high in soy and low in fat, unlike the Western diet. Interest in the protective effects of soy derivatives has led to the research of phytoestrogens and metabolites of soy that are described by some as natural SERMs. As a result, many clinical questions have been raised as to whether phytoestrogens, which are also found in other natural foods, can protect against breast cancer. This article reviews the development and role of the more common SERMs, tamoxifen and raloxifene. In addition, this paper will also highlight the emerging studies on phytoestrogens and their similarity and dissimilarity to SERMs.

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Introduction

Great strides have been made in the last 25 years in the fight against breast cancer. One of the more notable developments has been the search for ways to prevent cancer. The development of selective estrogen receptor modulators (SERMs) has been a significant step towards achieving that goal. Tamoxifen, an antiestrogen in the breast and the pioneering SERM, has been the gold standard, and often the only choice in many countries for the treatment of breast cancer [1]. It also became the first drug ever to be approved by the United States (US) Food and Drug Administration (FDA) for the chemoprevention of breast cancer in high-risk women [1]. This chapter will review the development of tamoxifen the prototypical SERM and its use and development as a chemopreventive agent. In addition this article will also highlight the emerging information regarding phytoestrogens that are being regarded by some as natural SERMs.

Background

By the turn of the 20th century it was known that oophorectomy in pre-menopausal women with metastatic breast cancer could cause regression of the disease [2], [3]. This showed a link between products produced by the ovaries and the growth of some breast cancers. The product was found to be estrogen [4]. In 1936, Professor Antoine Lascassagne hypothesized that breast cancer was caused by a special hereditary sensitivity to estrogen and suggested that the development of an estrogen antagonist could prevent disease [5]. Over twenty-five years later in 1962 Jensen and Jacobsen [6] described the estrogen receptor (ER) as the mediator of estrogen action, setting the stage for the manipulation of this receptor for multiple purposes [7].

Investigation of possible contraceptive agents led to the reinvention of ICI 46 474, a failed contraceptive agent, to become tamoxifen, the first targeted anti-cancer agent. The study of tamoxifen

Jordan, V.C.

in the laboratory led to the finding that it inhibited the growth of ER-positive breast cancer cells *in vitro* [8]. In addition, animal studies showed that tamoxifen prevented rat mammary carcinogenesis [9], [10] but had a stimulatory effect on rat uterine weight [11]. The actions of non-steroidal antiestrogens were clearly not wholly explainable as estrogen agonists or antagonists and a model to describe their unique actions led to the development of the SERM concept [12], [13], [14].

What are SERMs?

SERMs are synthetic non-steroidal agents that bind to the ER and produce a change in the biological activity of the receptor depending on the tissue type. The primary target site for SERMs, the ER, is a nuclear receptor. To fully understand the unique nature of SERMs the actions of estrogen on the body must be revisited. Estrogen in premenopausal women is primarily produced by the ovaries. There are multiple target sites for estrogen and it has various actions throughout the body. Estrogens decrease cholesterol levels by lowering the circulating low-density lipoproteins (LDL). Its actions also include maintenance of bone density in postmenopausal women, and hormonal regulation, and control of the menstrual cycle in premenopausal women. These actions are summarized in ▶ **Fig. 1**. In contrast, the effect of SERMs depends on the target sites and is shown in ▶ **Fig. 2**.

A pure estrogen agonist would be one that stimulates the positive action of estrogen at all its targets. Conversely, a pure antagonist would inhibit all the actions of estrogen at all of its target sites. In contrast, SERMs have partial agonist and antagonist properties depending on the target site hence their uniqueness.

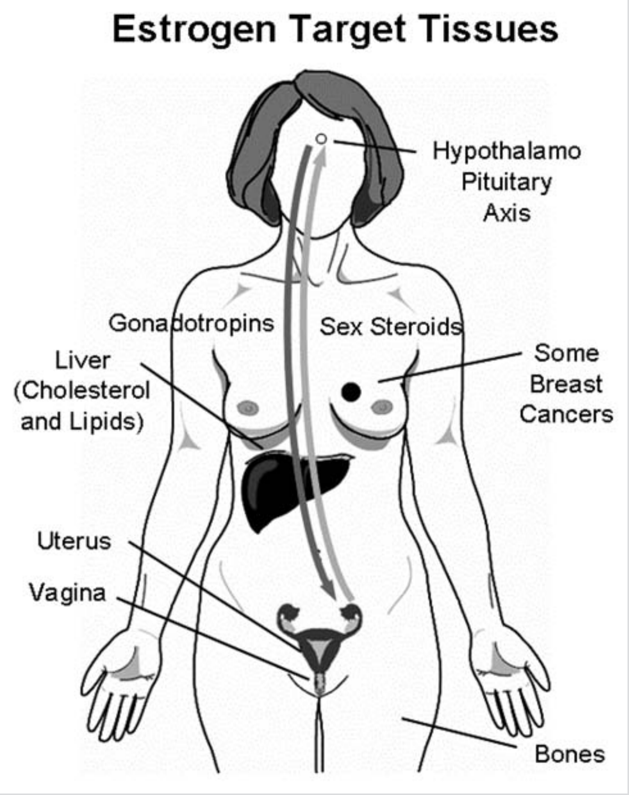

Fig. 1 The sites of action for estrogen.

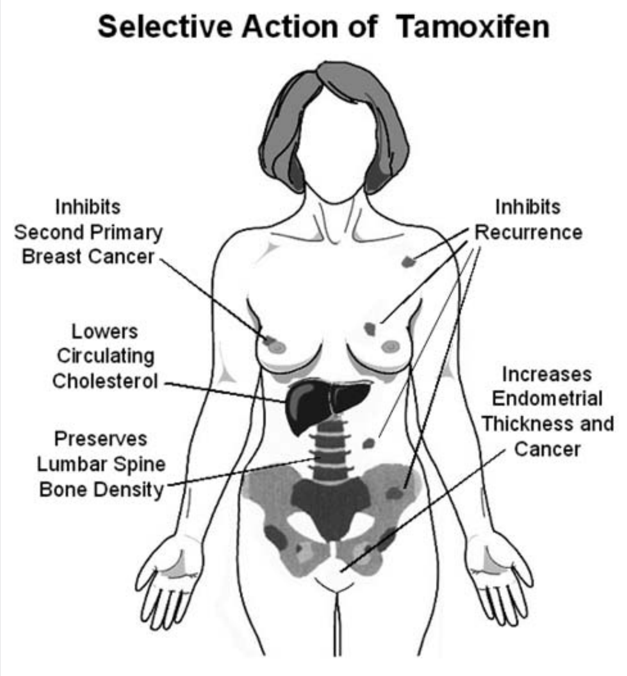

Fig. 2 The sites of action of tamoxifen.

Studies have shown that the partial agonist/antagonist properties depend on which associated coregulators are expressed when the receptor/ligand interaction occurs [15]. The details of the receptor/ligand interaction help us understand the mechanism of action of SERMs.

Mechanism of action

There are two aspects to the mechanism of action of SERMs: the pharmacokinetics or how the drug gets to the target site and the pharmacodynamics or what it does when it gets there. Tamoxifen (▶ **Fig. 3**) is a lipophilic prodrug that is easily absorbed by the gut without modification and 98 % is bound to albumin after entering the circulation. It undergoes extensive metabolism in the gastrointestinal (GI) tract and in the liver into its less active form *N*-desmethyltamoxifen and two most active forms, 4-hydroxytamoxifen and endoxifen [16], [17], [18], [19]. Each of the hydroxylated metabolites results from first pass metabolism in the liver. These compounds enter the bloodstream via the enterohepatic circulation to reach their target sites [18], [20], [21]. The metabolites of tamoxifen are excreted via the fecal route as has been shown by animal studies using ^{14}C radiolabeled tamoxifen [22]. These studies demonstrate that 67 % of these metabolites enter the enterohepatic circulation and undergo further metabolism several times until excretion by the GI tract [23], [24]. 4-Hydroxytamoxifen, and endoxifen have the same affinity for the ER as estrogen. Other metabolites of tamoxifen do not have as much effect or affinity for the ER as they lack the 4-hydroxy group [18]. Recent studies demonstrate that the potent tamoxifen metabolite endoxifen is produced by the product of the CY2PD6 gene. In patients with mutations of the CYP2D6 gene or patients who take other medications that compete for the enzyme product, metabolism of tamoxifen to the potent metabolite endoxifen is affected and may therefore have less benefit [25], [26]. Raloxifene (▶ **Fig. 3**), another SERM, is a polyphenol, which undergoes rapid conjugation in the GI tract and in the liv-

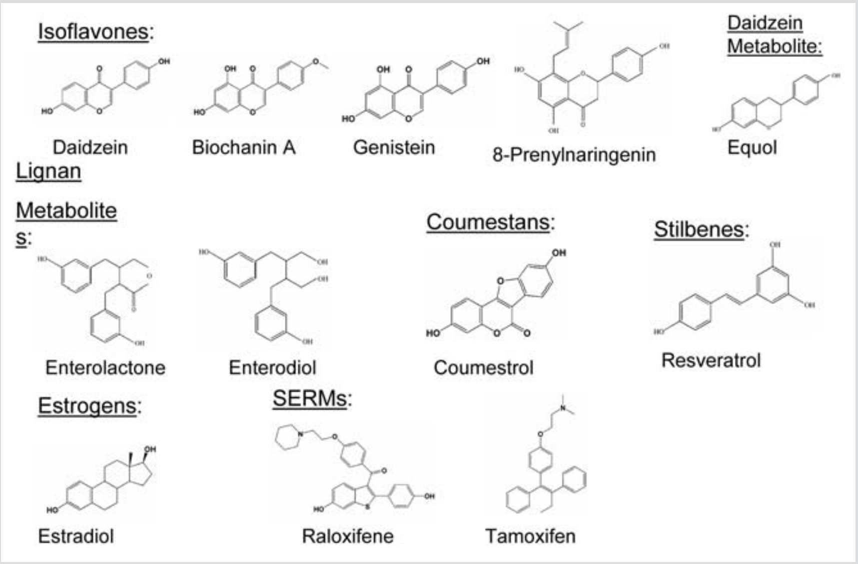

Fig. 3 A structural comparison of commonly studied phytoestrogens and phytoestrogen metabolites to SERMs.

er. In addition it also undergoes phase 3 metabolism by gut flora. The bacteria directly glucuronidate and sulfate this compound so that it is excreted [26], [27]. Since the drug does not reenter the enterohepatic circulation, it does not reach its targets as efficiently as tamoxifen. Also, a smaller percent enters the circulation as only 2% is bound to albumin and the half-life of raloxifene is 27 hours [28]. As a result of differences in metabolism and bioavailability, raloxifene is not as useful an agent in patients who already have breast cancer [29].

There are two isoforms of the ER, ERα and ERβ [6], [30] whose distribution and density varies depending on the target site. Both isoforms are found in the reproductive organs. Tamoxifen binds both receptors with equivalent affinity [31]. Endoxifen and 4-hydroxytamoxifen have similar affinities for both isoforms [32] and create similar gene expression profiles. Other ligands show preference for one isoform or the other, which may explain specific target tissue responses with various compounds. In many tissues, ERβ has anti-proliferative effects, whereas, ERα has proliferative effects [33]. Studies indicate that ER-β has an inhibitory effect on ER-α [34], [36], [35]. However, the biology is more complex than a simple agonist/antagonist interaction between the two receptors. The ratio of ERα to ERβ at a target site may be important in determining the overall action of a SERM on that tissue. A high ratio may correlate with high levels of cellular proliferation while a low ratio implies the opposite [36].

In the past, the interaction between SERMs and the ER was thought to be a simple case of a ligand switching its target receptor on or off. Through further research it is now known that this interaction is a more complex and dynamic process. Studies using phage display created a fingerprint of exposed surfaces when tamoxifen or estrogen was bound to the ER. Different conformational changes occur in the ER depending on the ligand that binds to the ER. In addition, the fingerprint was different in ER-α vs. ER-β when they were bound to identical ligands [37]. The discovery of the steroid receptor co-activator protein (SRC1) helped further to elucidate this complex interaction [40]. The binding of an SERM to the ER results in a conformational change in the ER [41], which results in the exposure of different amino acids on the receptor and the binding of different coactivators. Since the discovery of SRC1, dozens of other co-activator and co-repressor molecules have been discovered; all of which play some role in receptor modulation [15].

Finally, another dimension of signaling pathways can modulate the ER. Activation of the ER by other growth factor pathways can result in resistance to SERMs in a tumor.

This recruitment of specific co-regulators to the ligand receptor complex depends on the ligand that binds to the ER, the ER isoform, and “cross-talk” with other growth factor pathways [38]. SRC-3 is known to be important as a co-activator in breast cancer. In tumors and cancer cell lines that are HER2-positive and resistant to endocrine therapy with tamoxifen, studies demonstrate that SRC-3 is recruited to ER-α, but not ER-β in the presence of tamoxifen. In specimens from patients who were HER-2-negative and sensitive to endocrine therapy with tamoxifen, estrogen recruited SRC-3 to both ER isoforms, but tamoxifen did not [42]. Finally, when SRC-3 was knocked down, there was reduced expression of the estrogen target gene, pS2 in MCF7 cells. After the SRC-3 knockdown in cells derived from HER2-positive tumors, there was a decrease in cell proliferation and the cells regressed in the presence of tamoxifen [42].

To summarize the molecular process thus far: once an SERM binds to the ER it causes a change in the shape of the ER. This change of shape allows recruitment of co-activators, if it is destined to elicit an estrogenic response, or co-repressors if its response is anti-estrogenic. The binding of the coregulatory molecules leads to the activation of the promoter sequence of the estrogenic responsive gene [36]. This process is also controlled by the degradation and disassembly of complexes at the gene promoter site, which causes renewed activation of the signal to initiate RNA synthesis. In this way the SERM can specifically modulate the estrogen responsiveness of a target tissue (See review Jordan [36]).

Clinical relevance

The full details of the mechanism of action of SERMs have yet to be precisely described however, their clinical importance as an advance in medicine is proven. Tamoxifen was initially tested in humans in the early 1970 s, before extensive anti-tumor testing in animals [39], [40]. Animal testing [1], [9], [10] refocused efforts and targeted the ER [41], thereby opening the door for chemoprevention. Through animal studies tamoxifen was found to have targeted anti-tumor activity and initially, anti-estrogenic activity correlated with anti-tumor activity. These findings led to extensive human trials that helped consolidate the actions of SERMs and refined their applications. In initial human studies tamoxifen, an “antiestrogen”, was found to lower bone density in pre-menopausal women [42]. However, the “estrogen-like” actions of tamoxifen, maintained bone density in post-menopausal women [43], [44]. In the uterus tamoxifen acts as an agonist and increases the risk of endometrial cancer in post-menopausal women [45]. The next sections review the large-scale human chemoprevention trials of SERMs.

Chemoprevention

The first large human trial involving tamoxifen was the Royal Marsden study performed by Powles and colleagues [46], [47]. For this study approximately 3000 high-risk women were recruited and randomized to receive treatment with tamoxifen 20 mg/day for 8 years or placebo. High-risk status was determined by family history and a history of benign breast disease. The study found a decrease in LDL and loss of bone density in premenopausal women, but increased bone density in postmenopausal women and increased endometrial thickening on ultrasound study. Although this study initially showed no difference in the incidence of breast cancer, it was not powered to detect a difference in the development of breast cancer with either treatment group. Nevertheless, the twenty-year follow-up of this study does show a statistically significant reduction in the incidence of ER-positive breast cancer in the tamoxifen treatment arm after the 8 years of treatment [48].

The National Surgical Adjunctive Breast and Bowel Project (NSABP) P-1 trial by Bernard Fisher and colleagues was the first major chemoprevention trial in the Unites Stated with tamoxifen [49]. Over 13,000 women were recruited for this study in multiple centers around the US and Canada. Once again high-risk status was determined by family history, breast biopsy with pathological findings of lobular carcinoma *in situ* or atypical ductal hyperplasia, no children, menarche by 12 and age at birth of first child of over 30. The initial results of the NSABP trial showed a 49% reduction in the risk of invasive breast cancer and a 50% reduction in the risk of non-invasive breast cancer. Tamoxifen also reduced the incidence of osteoporotic fractures. No difference was seen in the risk of myocardial infarction but there was an increased risk of deep venous thrombosis, endometrial cancer and cataracts in the tamoxifen group.

Based on these clinical trials in 1998, tamoxifen was approved by the US FDA for reduction of the risk of breast cancer in high-risk women. Despite the positive results of the NSABP P-1 trial the side effects noted in the tamoxifen group resurrected the interest in other SERMs that had similar chemopreventive profiles to tamoxifen but with a more desirable side effect profile. This has led to human trials with raloxifene, an old compound, which had not been studied much since its discovery in the late 1970 s [50], [51].

Prevention of osteoporosis

In laboratory studies raloxifene was shown to inhibit DMBA-induced rat mammary carcinoma growth [52] and development [53], however, it was not as potent as tamoxifen. More importantly, raloxifene was as effective as tamoxifen in maintaining ovariectomized rat bone density but was less estrogen-like than tamoxifen in the rodent uterus [13] or in stimulating mouse endometrial tumor growth [54]. The short half-life of raloxifene makes it a difficult drug to dose, nonetheless; clinical trials with raloxifene have also helped define its pharmacology. The Multiple Outcomes for Raloxifene Evaluation (MORE) trial evaluated the effects of raloxifene in postmenopausal women [55], [60]. This study was extended to eight years as the Continuing Outcomes Relative to Evista (CORE) trial [61]. The results of the MORE/CORE trials demonstrated the effectiveness of raloxifene in preventing osteoporosis. In addition, raloxifene also inhibited the development of invasive breast cancer by 65% [61]. These clinical data justified the evaluation of raloxifene against tamoxifen to reduce the risk of breast cancer in high-risk postmenopausal women. The Study of Tamoxifen and Raloxifene (STAR) trial, was a phase III double-blinded study that randomized eligible postmenopausal women at a high risk for breast cancer, to receive tamoxifen 20 mg daily or raloxifene 60 mg daily [56]. The STAR trial demonstrated the equivalence of raloxifene and tamoxifen in reducing the incidence of invasive breast cancer. Furthermore, raloxifene had a better side effect profile with a lower incidence of endometrial cancer and hyperplasia, deep venous thromboses and cataracts. A drawback of raloxifene, however, was its decreased effectiveness in preventing the development of non-invasive breast cancer after two years, when compared to tamoxifen. Currently raloxifene is FDA-approved for the treatment and prevention of osteoporosis, and risk reduction for breast cancer in high-risk postmenopausal women.

Extending chemoprevention

The development of a chemopreventive agent such as tamoxifen but which has significant side effects had led to interest in whether naturally occurring compounds have similar chemopreventive effects. Epidemiologic observations have made this question even more seductive. While the etiology may be unclear, it has been well documented that Asian women have a lower incidence of breast and colorectal than Caucasian women [57]. Asian diets in particular are high in soy foods, which are felt to be responsible for this difference. When Asian women emigrate to western countries their incidence of breast cancer approaches that of the indigenous population [58]. This phenomenon has been observed in Japanese and Caucasian women who emigrate to the United States. It has also been observed that the risk of breast cancer in Asian Americans decreases in relation to increasing intake of soy derivatives [59]. Additionally, Chinese women who adopt a more westernized diet also appear to increase their incidence of breast cancer. All these findings have generated an interest in soy foods and its impact on hormone levels in the body. Phytoestrogens are the focus of current investigations. However, it should be stressed at the outset that despite beliefs of benefits from changes in diet and administration of supplements, there are dangers that breast cancer growth could be enhanced rather than prevented.

What are Phytoestrogens?

▼

Phytoestrogens are plant derivatives that bear a structural similarity to 17-beta-estradiol and act in a similar manner. Structures of common phytoestrogens, SERMs and 17-beta-estradiol are shown in ▶ **Fig. 3**. The principal phytoestrogen groups are flavonoids, lignans, coumestans and stilbenes [60], [61], [62]. Phytoestrogens are present in common foods such as soybeans, grains, fruits and vegetables. An in-depth review of the various types of phytoestrogens is beyond the scope of this article, however, common properties of most phytoestrogens include their metabolism by gut flora to additional derivatives with varying estrogenic activity. Many studies have focused on isoflavones, which are a subgroup of the flavonoids, they include but are not limited to genistein, daidzein and biochanin A. These isoflavones have varying estrogenic activity [63] and isoflavones have been proposed as natural SERMs. Studies show that isoflavones act as antioxidants *in vitro* and exert antiproliferative activities [64], [65]. Equol (▶ **Fig. 3**), an estrogenic metabolite of the isoflavonoids family [66], is produced from daidzein by the action of intestinal flora. This metabolic conversion however occurs in only 30% of the population [67].

Lignans, the most prevalent phytoestrogens in the diet are found in whole wheat, fruits and vegetables. Lignans are metabolized by the action of gut microflora into enterolactones and enterodiol [60] with very weak estrogenic properties [66]. While there are many studies on isoflavones, there are significantly fewer studies on coumestans and stilbenes. Coumestans are potent activators of the ER signaling pathway but are not as prevalent in the diet. Resveratrol is the most common stilbene and its use as a chemopreventive agent against breast cancer is actively being studied in rodent models [60]. In the next section we will consider the mechanism of action of phytoestrogens. The interaction of phytoestrogens with ERs is in some ways similar to the SERM/ER interaction, but there are significant differences that confound biological comparisons.

Mechanism of action of phytoestrogens

Hydroxylated SERMs in general have a higher binding affinity for both ERα and ERβ compared to phytoestrogens. As with SERMS, phytoestrogens can bind to either ERα or ERβ however, phytoestrogens appear to have a higher affinity for ERβ [68]. This affinity may be dose-dependent but overall phytoestrogens have a significantly lower affinity for the ER than estradiol [69], [70]. In addition the estrogenic potency of phytoestrogens varies within the particular phytoestrogen group. For example, within the flavonoid family genistein has greater potency than biochanin A, which has greater potency than daidzein [63]. Kuiper and colleagues [31] demonstrated that the stimulation of transcriptional activity by both subtypes of the ER vary depending on the estrogenic potency of the phytoestrogen and the further use of reporter gene assays demonstrate that synthetic estrogens and phytoestrogens have varying affinity for the ER and for each ER isoforms [68].

SERMs are non-steroidal estrogens that become antiestrogenic by virtue of their correctly positioned side chain. However, the antiestrogen side chain is not present in phytoestrogens and this structural deficit may therefore limit their classifications as SERMs. Nevertheless, the presence of a correctly positioned phenolic ring and also the distance between the two opposing phenolic oxygens in the isoflavone structure is similar to that of 17-beta-estradiol (▶ **Fig. 3**). This similarity allows the isoflavones to bind to either subtype of ER, effectively displacing 17-beta-estradiol. Studies have found that isoflavones have both agonistic and antagonistic effects, although they are strong ERβ agonists and weak ERα agonists [71]. It is this pharmacological receptor interaction rather than competitive interaction at a single receptor site that may be responsible for some of the diverse biological actions of phytoestrogens. This action may explain how phytoestrogens protect against breast cancer, because ERβ inhibits mammary cell growth as well as the stimulatory effects of ERα [72]. However, there is yet another dimension of molecular action at the ER that might be important. It is not certain whether isoflavones displace the estradiol by binding to a primary site on the ER, causing competitive binding between the isoflavones and the estradiol, or whether the isoflavones bind to a secondary site on the ER [73]. In contrast, genistein has been found to bind to the active site of ERβ [74].

Recent studies have attempted to decipher the actual role of each receptor subtype in gene activation and physiological response. Part of the problem in determining the physiological actions of phytoestrogens is our ignorance of the actual role of the ERα and ERβ. For example, a study by Hertrampf and colleagues [75] shows that the osteoprotective effect of genistein is mediated through the ERα-dependent pathways and its effect is enhanced by physical activity. Also, the activation of ERβ may modulate ERα-mediated physiological effects *in vivo*.

Many factors such as the ligand, dose and interaction of the ligand and receptor all influence ER molecular biology at the target site [76].

As with the SERMs, studies have shown that the recruitment of coregulatory molecules may be important in determining the function of phytoestrogens. In particular, isoflavones appear to selectively trigger ERβ transcriptional pathways, especially transcriptional repression. This affinity for the ERβ results in the exposure of a weak activation function-2 (AF-2) on the surface of ERβ, which has greater affinity for certain coregulators compared to ERα [72]. Phytoestrogens also have differential activity on several ER associated signaling pathways. For example, Akt, which is normally phosphorylated secondary to activation of ERα, is up-regulated by genistein and daidzein in ER-positive breast cancer cell lines, while resveratrol has an inhibitory effect on the phosphorylation of Akt [77]. In contrast, in ER-negative cell lines, resveratrol and daidzein activate Akt and genistein inhibits activation of Akt [77]. This is clearly a non-ER event, but whether this is cancer-specific or a toxicity of studies conducted *in vitro* can only be resolved with studies *in vivo*.

Although the isoflavones have agonistic and antagonistic estrogenic effects, the phytoestrogens also induce differentiation as well as inhibit angiogenesis, cell proliferation, tyrosine kinase, and topoisomerase II; all of which will help prevent tumor growth. However, it is important to stress again that despite the fact that there have been numerous and extensive laboratory studies on the mechanisms of breast cancer chemoprevention with phytoestrogens, there is no definitive evidence that proves that phytoestrogens are chemopreventive but they may contribute to adverse outcomes in breast cancer [78].

Cell and animal studies on the effect of phytoestrogens

Phytoestrogens have been likened to natural SERMs, and a brief survey of cell and animal studies of phytoestrogens reveals some similarities to SERMs such as tamoxifen. The approach to these studies may be classified into three broad categories. The first are studies that focus primarily on the role of phytoestrogens as a chemopreventive agent. The second are those studies that focus on phytoestrogens as a treatment agent. The third are those studies that focus on the biological effects when phytoestrogens are used continuously from neonates to adults.

The first category focuses on the chemopreventive effects of phytoestrogens in animal models that are subsequently treated with a chemical carcinogen. Animal studies have shown that when rats are treated with phytoestrogens and then exposed to a carcinogen they are less likely to develop breast cancer if exposure to phytoestrogens occurs at an early age [79], [80]. Lamartiniere and colleagues [79] demonstrated that the timing of exposure to phytoestrogens whether pre- or post-puberty, may influence their action on preventing mammary carcinogenesis. Lamartiniere [79] found that neonatal injections of genistein reduced the incidence of DMBA-induced mammary tumors in rats. Further evaluation revealed that the overall effect of genistein on prepubertal rats appeared to be secondary to early differentiation in mammary tissues resulting in less active EGF signaling pathways in adulthood that may be protective against breast cancer. A recent meta-analyses by Warri et al. [81] revealed that pubertal exposure to phytoestrogens result in changes in the mammary gland morphology and signal pathways that mimic those induced by the estrogenic environment of early first pregnancy.

The second group of studies focus on the use of phytoestrogen treatments in both tumor-implanted athymic mice and breast cancer cell lines. Studies have shown that treating estrogen-sensitive MCF-7 cell lines with genistein has an inhibitory effect on their growth [82]. However, not all studies have had such conclusive findings such as that the action of phytoestrogens on breast cancer cells may be dose-dependent. At low concentrations phytoestrogens may stimulate growth, and at high concentrations inhibit growth [66], [82], [83], [84], [85]. The studies by Helferich help elucidate the dose-dependent actions of isoflavones [93], [86]. In animal studies, in which ovariectomized athymic mice were implanted with MCF-7 cells, genistein promotes the growth of ER+ MCF 7 cells and the effect of this isoflavone was dose-dependent. At concentrations as low as 10 nM genistein promoted growth of ER-dependent MCF-7 cells *in vitro* [86]. At higher concentration (> 20 microM) genistein inhibited the MCF-7 cell growth. In addition genistein can stimulate growth of MCF-7 cells *in vivo* in a dose-dependent manner [87]. Clearly, these data call for caution with the use of phytoestrogens in women with breast cancer.

Indeed, the early study by Welshons et al. [66] cautioned against the use of antihormonal therapies that did not block the ER for the treatment of breast cancer because high fiber or exclusively vegetarian diets with phytoestrogens-containing food supplements could enhance the probability of tumor recurrence and growth. Furthermore the combination of phytoestrogens and tamoxifen to treat breast cancer may result in decreased efficacy of tamoxifen. In a study evaluating the development of tumor and the tumor latency period, tamoxifen-treated mice fed a low dose isoflavone-enriched diet had a higher tumor incidence and a shorter tumor latency period than placebo-treated mice [95]. In addition tamoxifen-associated mammary tumor prevention was also significantly reduced. Nevertheless, certain phytoestrogens have also been noted to cause apoptosis of human breast cancer cells and this occurred at concentrations of 20 – 25 micromol/L [88], [89], [90]. While phytoestrogens have been observed to cause these various actions *in vitro*, it is unclear that *in vivo* the concentrations needed to achieve these actions are attainable. In animal studies a protective effect of phytoestrogens on the development of mammary cancer are conflicting [91], [92]. Santell and colleagues [92] have shown that while genistein may inhibit breast cancer cells *in vitro*, treatment of tumor-bearing athymic mice with genistein did not inhibit tumor growth, however in their study ER-negative human breast cancer cell lines were used. It would seem that the ability of phytoestrogens to be toxic *in vitro* at high concentrations does not extrapolate to models *in vivo* where the ability to maintain high local concentrations for long periods may be impaired.

A third approach is the study of the effects from early exposure to phytoestrogens from the perinatal periods and onwards. This approach was recently adopted by Mardon and colleagues [93]. Rats perinatally or lifelong exposed to a rich isoflavone diet exhibited higher body weight and fat mass at 24 months of age. Perinatal exposure to phytoestrogens led to higher bone mineral density in later life [93]. The translation of these data to human epidemiology and pharmacology is the challenge and has no immediate application to effects on mammary carcinogenesis. The observation is an estrogen-like action on bone rather than SERM related.

Human trials

Human trials on phytoestrogens differ from SERMs because unlike the SERMs, there are no major large-scale prospective studies of chemoprevention and pharmacology. Human studies on phytoestrogens can be divided into two broad categories. The first are studies that evaluate the effect of phytoestrogens on estrogen biosynthesis and excretion, the second are those studies that evaluate the overall impact of dietary phytoestrogens on specific clinical endpoints such as menopausal symptoms and bone mineral density presumably through a stimulatory action through the ER. Many studies have examined the use of phytoestrogens as chemopreventive agents; however, these studies are of limited value as they are retrospective.

Estrogen biosynthesis and excretion

Human studies on the effect of phytoestrogens on estrogen biosynthesis and excretion usually evaluate levels of circulating estrogen or steroid by-products and metabolites in the urine. In addition in many of these studies the levels of phytoestrogens are also measured and factors that affect these levels are explored. Human studies have shown conflicting results regarding the overall effect of phytoestrogens. Lu and colleagues [94] treated 10 pre-menopausal women with a soy-containing diet beginning on day two of the menstrual cycle to day two of the next cycle. Blood and urine samples were obtained before and during the initiation of the soy diet. Their results showed that the circulating levels of 17-beta-estradiol decreased by 25 %, however, cycle length did not change [94]. A dietary intervention study by Kumar and co-workers showed similar findings [95]. This study randomized women to receive 40 mg of isoflavones day or placebo for a 12-week period. They found that serum free estradiol and estrone levels decreased. Serum hormone binding globulin increased and mean cycle length also increased. Conversely, a year-long dietary intervention study by Maskarinec and co-workers [96] in premenopausal women did not find any difference in cycle length or hormone levels. These studies raise the question that while dietary intake of phytoestrogens is important, intake alone may not be the determinant of a chemoprotective effect.

Since a Finnish case control study [97] suggests that high enterolactone concentrations are associated with decreased breast cancer risk., it is possible that lifestyle factors that affect enterolactone may be linked to breast cancer risk. Whether these lifestyle factors that control enterolactone levels are linked to breast cancer risk remains to be seen. Administration of antibiotics has been noted to decrease the serum concentration of enterolactone for a prolonged period [98]. Premenopausal women who are treated with long-term antibiotics for urinary tract infections seem to be at higher risk for breast cancer, presumably because it alters the gut metabolism of phytoestrogens [99]. Smoking and obesity have been noted to decrease plasma enterolactone levels, however, tea, coffee, fiber and vegetables have the opposite effect [100]. In a study monitoring plasma enterolactone levels, women were noted to have a higher plasma concentration while on wheat bread 41.1 nmol/L compared to 15.4 nmol/L while on white bread [67]. Links to actual cancer risk do not exist but associations have been noted.

In human studies, it is often difficult to measure serum levels of phytoestrogens, because of a short half-life. Since most phytoestrogens are excreted in the urine, urine analysis of metabolites of phytoestrogens can be used to give an indication of exposure to phytoestrogens [101]. Urinary excretion of phytoestrogens varies in different regions of the world [102]. Women in areas with a low incidence of breast cancer have higher urinary isoflavonoids than women living in areas with a high incidence of breast cancer. Vegetarians also have a higher concentration of isoflavonoids in their urine than omnivores [103]. The excretion of equol in the urine has been proposed as a possible marker of the chemoprotective effect of phytoestrogens [112], [113]. Duncan and colleagues [104] studied the hormone profile of equol excretors versus equol non-excretors and found that regardless of the amount of phytoestrogens ingested in the diet, equol excretors had decreased levels of estrone, estrone sulfate, testosterone, DHEA and higher levels of steroid hormone binding globulin. This steroid hormone profile has been found to be a protective profile for breast cancer. The possible mechanisms to create a “change profile” may include the findings that phytoestrogens stimulate the production of sex steroid binding globulin by liver cells [103] and have inhibitory effects on the enzymes involved in the synthesis of estrogen. Phytoestrogens are known to decrease the conversion of androgens to estrogen by blocking the aromatase enzyme system. [105].

Phytoestrogens and cinical endpoints

The second group of human studies are those that focus on the effect of phytoestrogens on focal clinical endpoints. These endpoints vary and include alleviation of menopausal symptoms, maintenance of bone mineral density and development of breast cancer in some retrospective studies. Given recent concern regarding the possible adverse effects of hormone replacement therapy other alternatives for treatment of menopausal symptoms have been explored and phytoestrogens have played a significant role. A recent Cochrane review of the database revealed no clear evidence of the effectiveness of phytoestrogens in alleviating menopausal symptoms [106]. This notwithstanding, there are some small trials which show a benefit to using phytoestrogens for treating menopausal symptoms. In a double-blind prospective study sixty women were randomized to receive 60 mg of isoflavones daily for 3 months or placebo [107]. The menopausal symptoms before and after treatment were recorded. Women receiving the phytoestrogens treatment noted a 57 % and 43 % decrease in the incidence of hot flashes and night sweats, respectively. Similar results were seen in a small trial using a 6-week treatment of flaxseed for the treatment of menopausal symptoms [108]. Some investigators are evaluating the use of phytoestrogens as alternative agents to hormone replacement therapy (HRT) in the management of postmenopausal symptoms [107]. Recently, prenylated flavonoids derived from hops are being used to treat menopausal symptoms. One such compound is 8-prenylnaringenin (▶ **Fig. 3**) that has strong estrogenic activity [109]. MenoHop an agent containing the phytoestrogen 8-prenylnaringenin, is currently being evaluated to treat menopausal complaints in Belgium [110].

The relationship between phytoestrogens and bone health remains unclear, with some studies showing a benefit associated with phytoestrogen treatment and others showing none [111]. Supplementation of diet with isoflavones has been shown to help maintain lumbar spine bone density [122], [112]. A randomized double-blind control trial was performed to compare with HRT, the effect of the phytoestrogen genistein on bone metabolism and bone mineral density [113]. Patients were randomized to receive either HRT daily (1 mg of 17-beta-estradiol and 0.5 mg norethisterone) or genistein 30 mg daily or placebo daily for a period of 1 year. On completion of this protocol women receiving the HRT and genistein had significantly increased bone mineral density in the femur compared to those in the placebo group. In another randomized control trial, Atkinson and colleagues [114] showed that women receiving an isoflavones extract had a decreased loss of lumbar spine bone mineral content and bone mineral density compared to placebo.

Direct studies on the efficacy of phytoestrogens in preventing breast cancer are difficult given the length of time required to perform such a study. Indeed, this obstacle with phytoestrogen research illustrates how powerful SERMS are to produce dramatic decreases in breast cancer incidence within 5 – 10 years [55], [115]. However, surrogate endpoints such as the effect of phytoestrogens on breast cell proliferation and mammographic density have been studied. Increased breast cell proliferation and increased mammographic density are risk factors for malignancy. Short-term dietary supplementation with phytoestrogens stimulates breast epithelial proliferation [116]. This finding has also been noted in premenopausal women treated with prolonged phytoestrogen intake [117]. This breast proliferation is evident on mammograms as increased mammographic densities and some of these parenchymal patterns are associated with a higher risk of breast cancer [118]. These histological findings are supported by the observation of increased high risk parenchymal sonographic patterns in women who report low dietary soy protein intake [119]. Other studies such as that by Maskarinec and colleagues [120] show a similar finding in mammographic density in women treated with prolonged phytoestrogen supplementation.

As noted in animal studies, [101] the age at which a woman is exposed to phytoestrogens and length of exposure to phytoestrogens may be important in determining whether a protective benefit is obtained. A prospective 12-ear study of diet and breast cancer by Key and colleagues [121] of over 30,000 women in Japan showed that there was no relationship found between soy food consumption and the development of breast cancer, however this study was comprised of mostly non-adolescent women. In contrast, Shu and colleagues [122] performed a retrospective case controlled study on Chinese women with breast cancer. Subjects completed a questionnaire regarding their dietary intake in adolescence. A high soy consumption as an adolescent was associated with a decreased incidence of breast cancer as an adult. This may also explain why when women emigrate to countries with a higher incidence of breast cancer than their native country, they are more likely to have a decreased incidence of breast cancer if they emigrated after puberty [123].

While there is increasing excitement at the possible role of phytoestrogens as chemopreventive agents or as complimentary alternative medicine for menopausal symptoms their safety profile remains largely unknown and concerns regarding this have been raised in two recent reviews [124], [125]. Isoflavones such as genistein have been found to stimulate the growth of MCF-7 cells [86], [93]. Some studies have shown that soy products increase breast epithelial cell proliferation [125], [126], which may increase the risk of breast cancer. These findings suggest caution in the broad use of phytoestrogens. In addition the interaction of phytoestrogens and tamoxifen inbreast cancer patients may negate the protective effects of SERMs and caution has een advised against thebination of these two agents [126].

Conclusion

Since their discovery the use of SERMs in clinical practice continues to expand [127], [128], [129]. As our knowledge of phytoestrogens grows, so does our understanding of their interaction with the ER and ability to possibly act as a natural SERM or conversely to antagonize the actions of SERMs. However, based on their structure-function relationships, the molecular endocrinology of SERMs and phytoestrogens is very different and the phytoestrogens appear to act as ER agonists at low concentrations but may act as antagonists by biochemical mechanisms through the ER beta receptor complex. Despite the advances in the treatment of breast cancer, prevention if possible must be superior to treatment. Currently tamoxifen and raloxifene are the first important steps in the quest to develop a complete preventative agent. In the future, a role, if any for the phytoestrogens or their derivatives may emerge, but current research is too weak to provide any clinical guidelines beyond caution. Alternatively, clues from laboratory studies may prove to be important in future drug development. An example of this is the current interest in the pharmacology of resveratrol which may have valuable pharmacological actions not mediated via the ER [130], [131].

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Tyrosine Phosphorylation of the Nuclear Receptor Coactivator AIB1/SRC-3 Is Enhanced by Abl Kinase and Is Required for Its Activity in Cancer Cells[▽][†]

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Overexpression and activation of the steroid receptor coactivator amplified in breast cancer 1 (AIB1)/steroid receptor coactivator-3 (SRC-3) have been shown to have a critical role in oncogenesis and are required for both steroid and growth factor signaling in epithelial tumors. Here, we report a new mechanism for activation of SRC coactivators. We demonstrate regulated tyrosine phosphorylation of AIB1/SRC-3 at a C-terminal tyrosine residue (Y1357) that is phosphorylated after insulin-like growth factor 1, epidermal growth factor, or estrogen treatment of breast cancer cells. Phosphorylated Y1357 is increased in HER2/neu (v-erb-b2 erythroblastic leukemia viral oncogene homolog 2) mammary tumor epithelia and is required to modulate AIB1/SRC-3 coactivation of estrogen receptor alpha (ERα), progesterone receptor B, NF-κB, and AP-1-dependent promoters. c-Abl (v-Abl Abelson murine leukemia viral oncogene homolog 1) tyrosine kinase directly phosphorylates AIB1/SRC-3 at Y1357 and modulates the association of AIB1 with c-Abl, ERα, the transcriptional cofactor p300, and the methyltransferase coactivator-associated arginine methyltransferase 1, CARM1. AIB1/SRC-3-dependent transcription and phenotypic changes, such as cell growth and focus formation, can be reversed by an Abl kinase inhibitor, imatinib. Thus, the phosphorylation state of Y1357 can function as a molecular on/off switch and facilitates the cross talk between hormone, growth factor, and intracellular kinase signaling pathways in cancer.

Coactivators significantly enhance the rate of transcription by binding to, and bringing together, components of the basal transcriptional machinery complex at gene promoters. A member of the p160 steroid receptor coactivator (SRC) gene family amplified in breast cancer 1 (AIB1) (also called steroid receptor coactivator-3 [SRC-3], TRAM1, RAC3, ACTR, and NCOA3) is amplified, and its corresponding mRNA and protein levels are overexpressed in multiple cancers (3, 20, 29, 43, 58). Overexpression of AIB1/SRC-3 is associated with markers of poor prognosis in breast cancer cells, including exhibiting increased p53 expression, being HER2 positive, and lacking estrogen receptor (ER) and progesterone receptor (PR) expression (5, 38). Phenotypic studies strongly argue that AIB1/SRC-3 has a role in both hormone- and growth factor-dependent gene expression. Cancer cell line studies demonstrate that AIB1 is critical for growth dependent on estrogen (28) and insulin-like growth factor 1 (IGF-1); it protects cells against apoptosis or anoikis (a form of apoptosis that is induced by anchorage-dependent cells detaching from the surrounding extracellular matrix) (37) and increases cell size and proliferation (64). AIB1 also regulates epidermal growth factor (EGF) receptor tyrosine phosphorylation and the subsequent downstream EGF-induced activation of STAT5 and c-Jun N-terminal kinase (25). Targeted disruption of p/CIP (CREB-binding protein [CBP]-interacting protein), the mouse homologue of AIB1, demonstrates that AIB1 is critical for somatic growth (54, 59), energy balance (53), adipogenesis (30), and the rate of oncogene-induced (24) and carcinogen-induced (23) tumor formation. Overexpression of AIB1 or its naturally occurring isoform AIB1-Δ3 in mice caused increased mammary gland size, increased mammary epithelial cell proliferation (50), and increased tumor incidence in multiple organs (51).

Site-specific phosphorylation and dephosphorylation are common posttranslational modifications utilized to control target protein functions. For AIB1, serine and threonine phosphorylation has been described (57) and can be an initiating modification that occurs before further posttranslational modifications, e.g., sumoylation (55), ubiquitylation (16, 32, 56), or methylation (13, 33). How tyrosine phosphorylation regulates the interactions of AIB1 with these other modifying enzymes or with other transcription cofactors and its relationship to pathway signaling are examined here for the first time. Our study documents that a single, site-specific AIB1 phosphorylation (at Y1357) can change the interaction of AIB1 with three proteins often found in transcription complexes bound to promoter elements: a methyltransferase (coactivator-associated arginine methyltransferase 1 [CARM1]), a histone acetyltransferase (p300), and a nuclear receptor (estrogen receptor alpha [ERα]). Dynamic simulations suggest a molecular mechanism for these changed interactions postphosphorylation.

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For the first time, we demonstrate a novel role for c-Abl (v-Abl Abelson murine leukemia viral oncogene homolog 1) (Abl) kinase in steroid receptor signaling via alteration of coactivator function. Abl kinase directly phosphorylated and bound to AIB1 via the Y1357 site. These results suggest that there is an on/off switch for coactivating ability and that cross talk between steroid and growth factor signaling can occur in breast cancer cells via modulation of AIB1 Y1357 phosphorylation. Furthermore, detection of phospho-Y1357 is potentially a response marker in cancer tissues for inhibitors of Abl, such as imatinib (Gleevec).

MATERIALS AND METHODS

Plasmids and reagents. p300-HA, CARM1-HA, and c-Abl-AU5 plasmids were kindly provided by Maria L. Avantaggiati (Georgetown University), Michael R. Stallcup (University of Southern California), and J. Silvio Gutkind (NIH/NIDCR). AIB1-Δ3 plasmid was previously described (42). AIB1-Δ3-FLAG tag expression plasmids (wild-type, Y1357F, and S505A constructs) were made by PCR amplification of ACTR/AIB1-Δ3 cDNA (778 bp to 4,422 bp) to add a new 5′ NotI site and a 3′ BglII site. PCR product was cloned into p3XFLAG-CMV-10 (Sigma-Aldrich, Inc.). Imatinib (STI-571, Gleevec; Novartis, Inc.) was kindly provided by Jeffery A. Toretsky (Georgetown University). EGF was purchased from Roche Diagnostics Co. IGF-1 was purchased from R&D Systems.

Cell lines. MCF-7 and COS-7 cells were grown in Iscove modified Eagle medium (Invitrogen Co.) with 10% heat-inactivated fetal bovine serum (HI-FBS; Quality Biological Inc.). MDA-MB-231, A549, HeLa, and 293T cells were grown in Dulbecco modified Eagle medium (Invitrogen Co.) with 10% HI-FBS. CHO-K1 cells were grown in F12-Dulbecco modified Eagle medium (Invitrogen Co.) with 10% HI-FBS. Cells were hormone stripped in media containing 5% charcoal/dextran-stripped FBS (HyClone).

Immunoprecipitation (IP) and Western blot (WB) analysis. (i) IP experiments with MCF-7, A549, and MDA-MB-231 cells. Cells were grown to 80% confluence in 150-mm dishes, were serum starved for 24 h, and were untreated or treated with 50 ng/ml of IGF-1 or EGF for 10 min. Cells were washed with cold phosphate-buffered saline (pH 7.4) and harvested with 1% NP-40 lysis buffer containing 1 mM NaO_3VO_4 and 1× Complete protease inhibitor cocktail (Roche Diagnostics Co.).

(ii) IP experiments with 293T cells. 293T cells were transfected with 4 μg of each plasmid. The antibodies used for IP were 4G10 phosphorylated tyrosine antibody (Ab) agarose conjugate (Upstate Biotech, Inc.), AIB1 monoclonal antibody (MAb) (BD Transduction Laboratories), phospho-Y1357 AIB1 polyclonal Ab (Pacific Immunology Co.), FLAG M2 affinity gel (Sigma-Aldrich, Inc.), hemagglutinin (HA) affinity matrix (Roche Diagnostics), AU5 (Covance Co.), Abl (BD Biosciences), and ERα Ab-7 (Lab Vision Co.). IP was performed as previously described (25). Protein lysates were subject to NuPAGE gel electrophoresis (Invitrogen Co.).

(iii) WB analysis. Western blot analysis was done as previously described (37). Additional antibodies used for WB were phospho-CrkL Y207 (Cell Signaling Co.), ERα Ab-15 (Lab Vision Co.), and actin (Millipore Co.), and HA (Roche Diagnostics Co.) antibodies.

Phosphorylation mapping. (i) Sample preparation. Serum-starved MCF-7 cells were treated for 10 min with 50 ng/ml IGF-1 or EGF (R&D Systems). Whole-cell lysates were harvested with 1% NP-40 lysis buffer, precleared, immunoprecipitated with anti-AIB1 MAb (BD Transduction Laboratories), and run on a 4 to 12% sodium dodecyl sulfate-polyacrylamide gel (Invitrogen Co.).

(ii) Phosphorylation mapping by ProtTech Inc. Sequence grade modified trypsin (Promega Co.) or Asp-N (Roche Diagnostics) was used for protein digestion reactions. For each digest, ~20 to 50% of the sample was used for phosphatase differential analysis. Two aliquots of peptide mixture were analyzed for each digestion: one unit of alkaline phosphatase (Roche Diagnostics) was added to the treated reaction mixture, while in the control reaction, heat-inactivated alkaline phosphatase was used. Both samples were commercially analyzed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) (Micromass Proteome Work System MALDI-TOF Reflectron mass spectrometer). α-Cyano-4-hydroxycinnamic acid was used as a matrix. Phosphopeptides were identified by manually comparing the spectra from phosphatase-treated and control samples.

Luciferase reporter assays. Luciferase assays were performed as previously described (42) using the luciferase assay system (Promega Co.). A total of 3×10^4 hormone-stripped cells were plated in each well of a 24-well plate. Cells were transfected with FuGENE (Roche Diagnostics) for 16 to 24 h and then treated with hormones for 24 h. Cell extracts were prepared by using 100 μl of 1× passive lysis buffer (Promega Co.) and incubated at room temperature for 30 min on a rocker. Twenty microliters of the cell extract was assayed for firefly luciferase activity with the luciferase reporter assay kit (Promega Co.). Protein concentrations for each sample were determined using the Bradford protein assay. Luciferase values for each sample were normalized with their protein concentration.

Real-time reverse transcription-PCR. MCF-7 cells were transfected with AIB1 (3 μg) and ERα (0.5 μg) by electroporation (AMAXA kit V, program E-14) for 24 h. Cells were estrogen stripped and treated with 17β-estradiol (E2) (100 nM) for 3 h, and total RNA was harvested using RNA STAT (Tel-Test Inc.). One hundred fifty nanograms of RNA was used to perform real-time reverse transcription-PCR with the Platinum quantitative reverse transcriptase PCR ThermoScript one-step system (Invitrogen). Samples were reverse transcribed for 30 min at 56°C, followed by a denaturing step (3 min at 95°C) and 40 cycles (each cycle consisting of 15 seconds at 95°C and 1 min at 58°C). Fluorescence data were collected during the 58°C step (iCycler; Bio-Rad). pS2 (TFF-1 [trefoil factor 1]) probe and primers were purchased from Applied Biosystems (catalog no. Hs00170216_m1). The sequences of the beta-actin primers and probe were as follows: forward primer, 5′ CCT GGC ACC CAG CAC AAT; reverse primer, 5′ GCC GAT CCA CAC GGA GTA CT; probe, 5′ 6-carboxyfluorescein-TCA AGA TCA TTG CTC CTC CTG AGC-Black Hole Quencher (IDT DNA Inc.).

Site-directed mutagenesis. The QuikChange XL II mutagenesis kit (Stratagene Co.) was used to introduce amino acid mutations in pCDNA3-AIB1-Δ3 and pCMV-3XFLAG-AIB1-Δ3. The following primers (IDT Inc.) were used for the mutagenesis reaction: for Y1357F, sense, 5′phosphate-CCG CAG GCT GCA TCC ATC TTC CAG TCC TCA GAA ATG AAG GG; antisense, 5′phosphate-CCC TTC ATT TGT GAG GAC TGG AAG ATG GAT GCA GCC TGC GG. The mutagenesis reaction was performed under the following conditions using the RoboCycler 40. The PCR mixture contained the following: 5 μl of 10× QuikChange reaction buffer; pCDNA3-AIB1-Δ3 (200 ng); sense primer (100 ng); antisense primer (100 ng); 1 μl of deoxynucleoside triphosphate mix; 3 μl Quik solution. The PCR mixture was brought up to a volume of 50 μl. PCR cycling conditions were as follows: step 1 was 2 min at 95°C; step 2 consisted of 25 cycles, with each cycle consisting of 1 min at 95°C, 1 min at 60°C, and 30 min at 68°C for 30 min; and step 3 was 7 min at 68°C. The DNA from the mutagenesis reaction was digested with 1 μl of DpnI restriction enzyme for 1 h at 37°C to digest template DNA. Four microliters of the digested reaction mixture was transformed into 45 μl of β-mercaptoethanol-treated *Escherichia coli* XL-10 gold competent cells. Plasmid DNA was prepared, and DNA sequencing was performed to confirm mutation.

Phospho-antibody production. A rabbit polyclonal antibody to phospho-Y1357 AIB1 was raised against the phosphorylated peptide NH_2-SIpYQSSEM KGWPSGNLC-COOH (pY is phosphorylated tyrosine) (Pacific Immunology Co.). Titers against the phosphorylated and nonphosphorylated peptides were confirmed by enzyme-linked immunosorbent assays. Phospho-specific antibodies were purified sequentially using nonphosphorylated and then phosphorylated peptide affinity columns.

IHC. AIB1/SRC-$3^{-/-}$ (p/CIP$^{-/-}$) transgenic mice were previously described (59). FVB/N-TgN (mouse mammary tumor virus [MMTV]-HER2/neu) mice were purchased from Jackson Laboratory. Immunohistochemistry (IHC) analyses were performed on mammary gland 4 and tumor sections as previously described (50) using the phospho-Y1357 AIB1 rabbit polyclonal Ab. Briefly, tissues were fixed in 10% formalin and blocked in paraffin. Four-micrometer paraffin-embedded sections of mammary gland and tumor tissue were deparaffinized in xylene, rehydrated in alcohol, boiled for 10 min in citrate buffer (pH 6) (Zymed Laboratories) for antigen retrieval, and quenched with 3% hydrogen peroxide. The primary antibody was incubated overnight at 4°C. The phospho-Y1357 blocking peptide (Genscript) was prepared at four times the concentration of the phospho-Y1357 antibody. The peptide and antibody solutions were incubated together for 30 min at room temperature. The entire volume was added to the tissue section and incubated overnight at 4°C. Detection of rabbit primary antibodies were performed using the Dako Envision Plus horseradish peroxidase kit (Dako Cytomation). Bound antibody was visualized using diaminobenzidine substrate (Vector Laboratories). The slides were counter stained with hematoxylin (Polysciences, Inc.) for 30 s, dehydrated through an ascending concentration of ethanol, cleared in xylene, and mounted with Clearmount solution (Zymed Laboratories).

Protein modeling. (i) Structure prediction. Three-dimensional models of Y1357 were generated based on BLAST sequence alignment (1) with available crystal structures: 1SR9 (PDB annotation). Structure predictions for Y1357 were performed with the MODELLER 7v7 program (22).

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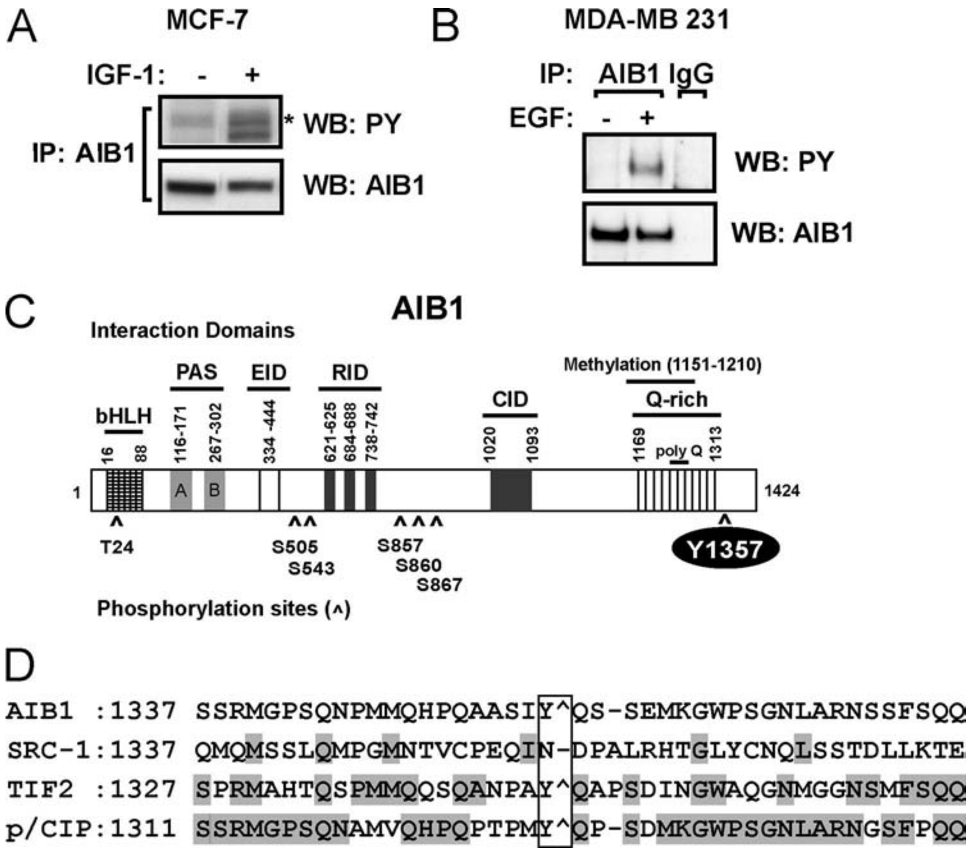
A
MCF-7
IGF-1: - +
IP: AIB1
* WB: PY
WB: AIB1
B
MDA-MB 231
IP: AIB1 IgG
EGF: - +
WB: PY
WB: AIB1
C
AIB1
Interaction Domains
bHLH
PAS
EID
RID
CID
Methylation (1151-1210)
Q-rich
16
88
116-171
267-302
334 -444
621-625
684-688
738-742
1020
1093
1169
poly Q
1313
A
B
1
1424
T24
S505
S543
S857
S860
S867
Y1357
Phosphorylation sites (^)
D
AIB1 :1337 SSRMGPSQNPMMQHPQAASIY^QS-SEMKGWPSGNLARNSSFSQQ
SRC-1:1337 QMQMSSLQMPGMNTVCPEQIN-DPALRHTGLYCNQLSSTDLLKTE
TIF2 :1327 SPRMAHTQSPMMQQSQANPAY^QAPSDINGWAQGNMGGNSMFSQQ
p/CIP:1311 SSRMGPSQNAMVQHPQPTPMY^QP-SDMKGWPSGNLARNGSFPQQ

FIG. 1. Growth factor-induced tyrosine phosphorylation of AIB1. (A) IGF-1 induced tyrosine phosphorylation of AIB1 in MCF-7 breast cancer cells. Cells were serum starved for 24 h and treated with 50 ng/ml IGF-1 for 10 min (+) or not treated with IGF-1 (−). Whole-cell lysates were harvested and used for immunoprecipitation (IP) and Western blot (WB) analysis with anti-AIB1 and antiphosphotyrosine (PY) antibodies as indicated. The asterisk indicates the position of a phosphotyrosine-containing band with a molecular mass of 165 kDa, which was confirmed to be AIB1 by reprobing the blot with the AIB1 antibody. The panel represents noncontiguous lanes from the same WB. (B) EGF induced tyrosine phosphorylation of AIB1 in MDA-MB-231 breast cancer cells. Cells were stimulated for 10 min with 50 ng/ml of EGF (+) and then processed and analyzed as described above for panel A. IgG, immunoglobulin G. (C) A schematic of AIB1/SRC-3 protein showing conserved and functional domains, serine and threonine phosphorylation sites, and the region containing multiple methylation sites. Phosphorylation at the Y1357 residue was discovered utilizing mass spectrometry. SRC-3 amino acid numbering was used for consistency. bHLH, basic helix-loop-helix; PAS, Per-Arnt-Sim; EID, E2F1 interaction domain; RID, nuclear receptor interaction domain; CID, CBP/p300 interaction domain. (D) Comparisons of amino acids surrounding Y1357 in AIB1 with other members of the p160 family, SRC-1, TIF-2/SRC-2, and p/CIP, the mouse homologue of human AIB1. Conserved amino acids are highlighted.

(ii) Energy minimization and molecular dynamics. The predicted wild-type and phosphorylated structures were energy minimized using the consistent valence force field (CF91) with default partial atomic charge available in Discover v3.0. Molecular dynamics simulations (300 ps) with distance-dependent dielectric constants were carried out using the SANDER module of the AMBER 7.0 suite programs (7) with PARM98 force field parameter (Accelyrs Inc.).

Abl in vitro kinase assay. Recombinant c-Abl kinase (80 ng) (Invitrogen Co.) was incubated with glutathione *S*-transferase (GST)-AIB1 (1017 to 1420 amino acids [aa]) (kindly provided by Don Chen, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School) purified from *E. coli* BL21 cell lysate. The reaction was performed for 30 min at 30°C in kinase buffer (50 mM Tris [pH 7.5], 10 mM $MgCl_2$, 0.01% NP-40, 1 mM dithiothreitol, 0.5 mM ATP). Phosphorylation was detected by Western blotting with pY1357 AIB1 polyclonal Ab.

Cell growth assays. Validated Abl small interfering RNAs (siRNAs) (exon 3, catalog no. 1346; exon 11, catalog no. 1431) were purchased from Ambion Co. and transfected as previously described (37). Hormone-stripped MCF-7 cells were plated in 1% charcoal-stripped calf serum and 10 nM ICI 182,780 (Tocris Biosciences) with or without 10 nM estrogen. Cell growth was measured by utilizing the WST-1 reagent (Roche Diagnostics) after 4 days.

Focus formation assays. AIB1/SRC-$3^{-/-}$ mouse embryonic fibroblasts (MEFs) were kindly provided by Jianming Xu (Baylor College of Medicine). A total of 2×10^6 SRC-$3^{-/-}$ MEFs were transfected with 2 μg of H-ras V12 and either 4 μg of empty vector, AIB1-Δ3 (wild type), or AIB1-Δ3 Y1357F constructs using the AMAXA MEF kit 2 (program A-23), plated in 100-mm dishes, and grown for 3 weeks with regular changes of the media. Plates were fixed with ice-cold methanol and stained with crystal violet (0.5% crystal violet, 25% methanol).

RESULTS

Tyrosine phosphorylation of AIB1 in breast cancer cell lines. We first investigated the change in overall tyrosine phosphorylation of AIB1 in MCF-7 breast cancer cells that had been treated with IGF-1. These cells were used because AIB1 is rate limiting for IGF-1 stimulation of their growth (37). AIB1 tyrosine phosphorylation was examined by immunoprecipitation of AIB1 from whole-cell extracts, and possible tyrosine phosphorylation of AIB1 was detected by Western blot analysis with an antiphosphotyrosine antibody (Fig. 1A). IGF-1 treatment increased by two- to threefold a phosphotyrosine-containing band with a molecular mass of 165 kDa, which was identified as AIB1 by reprobing the blot with the AIB1 antibody (Fig. 1A). We previously demonstrated that AIB1 is critical for EGF signal transduction in the MDA-MB-231 breast

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cancer cell line (25). Therefore, we asked whether EGF treatment of this cell line would also increase tyrosine phosphorylated AIB1 levels. We observed a significant increase in the phosphotyrosine AIB1 levels after 10 min of EGF stimulation (Fig. 1B). The blot was stripped and reprobed with the AIB1 antibody to confirm that this phosphotyrosine band was AIB1. These results demonstrate that growth factor-induced tyrosine phosphorylation of AIB1 is not limited to a single breast cancer cell line and that AIB1 can be tyrosine phosphorylated by both IGF-1 and EGF signaling pathway kinases.

Mapping of a phosphorylated tyrosine residue (Y1357) in AIB1. To identify specific growth factor-induced tyrosine residues in AIB1, we employed the mass spectrometry technique, MALDI-TOF. AIB1 in total lysates from IGF-1- and EGF-treated MCF-7 cells was immunoprecipitated with an AIB1 MAb. Samples were run on a sodium dodecyl sulfate-polyacrylamide gel, a band corresponding to AIB1 was excised, and its protein sequence was confirmed by a nano liquid chromatography-tandem mass spectometry technique before post-translational modification analysis was performed. After trypsin or Asp-N protease digestion, samples were analyzed by MALDI-TOF MS, and a phosphopeptide containing Y1357 was identified. In Fig. 1C, the location of Y1357 relative to previously identified serine/threonine phosphorylation sites is indicated (57). The major domains of AIB1 necessary for interaction with other transcriptional components are also indicated (3, 8, 13, 27, 31, 33, 49). The Y1357 site of SRC-3 is equivalent to ACTR Y1345, AIB1 Y1353, RAC3 Y1350, and TRAM-1 Y1357. The Y1357 site is located 67 aa proximal to the C terminus, juxtaposing a long polyglutamine tract (Fig. 1C) and is 264 aa distal to the C-terminal end of the CBP/p300 interaction domain. The Y1357 site and surrounding region has not been previously associated with any AIB1 functional domain. The Y1357 is also present in transcriptional intermediary factor 2 (TIF-2)/SRC-2 and in the mouse AIB1 homologue, p/CIP. Amino acids C terminal to the Y1357, notably Q and S residues, are also partially conserved in TIF-2/SRC-2 and p/CIP (Fig. 1D).

IGF-1 and EGF induce Y1357 phosphorylation in breast cancer cells. To confirm that the phospho-Y1357 site discovered by mass spectrometry analysis was phosphorylated in vivo, a rabbit polyclonal antibody was generated against a peptide containing the phospho-Y1357 residue and affinity purified. AIB1 was immunoprecipitated from MCF-7 total lysate with this phospho-specific polyclonal Y1357 antibody and the AIB1 MAb was used for WB analysis (Fig. 2A). Phospho-Y1357 levels were significantly upregulated (two- to fivefold) after either IGF-1 or EGF treatment in all three cell lines examined (Fig. 2A), indicating that the phosphorylation of Y1357 was not limited to a single cell line or growth factor. A 10- to 30-min treatment with either IGF-1 or EGF resulted in peak phospho-Y1357 levels, without changing the total amount of AIB1 protein (Fig. 2A, input panels) (see Fig. S3 in the supplemental material). It was previously shown that estrogen (E2) treatments can cause an increase in serine/threonine phosphorylation of AIB1 (57). We examined whether estrogen induces phosphorylation of Y1357 in both ERα-positive (MCF-7) and ERα-negative (MDA-MB-231) breast cancer cell lines. We found that phospho-Y1357 levels increased by approximately twofold after estrogen treatment without changing total AIB1 levels in MCF-7 cells (Fig. 2B, top MCF-7 panel). However, we did not observe estrogen-induced phosphorylation at the Y1357 site in MDA-MB-231 cells (Fig. 2B, top MDA-MB-231 panel). Our results indicate that exposure to IGF-1, EGF, or estrogen, in ERα-positive cell lines, can cause increased phosphorylation at Y1357 without changing total AIB1 protein levels.

Since we observed a robust increase in phospho-Y1357 levels in breast cancer cells by growth factor or estrogen treatment, we asked whether phospho-Y1357 could be detected in mammary tumors. To investigate this possibility, we examined by IHC the levels of phospho-Y1357 in mammary tumors that develop in the mouse mammary tumor virus (MMTV)-driven HER2/neu (v-erb-b2 erythroblastic leukemia viral oncogene homolog 2) transgenic mouse model. This model is strongly dependent on HER/ErbB receptor family signaling for proliferation and metastasis (17). In these tumors, we observed a significantly higher percentage of positive nuclei stained with the phospho-Y1357 antibody than in healthy mammary epithelial cells, indicating that AIB1 (p/CIP) is highly and selectively phosphorylated at residue Y1357 in these tumors (Fig. 2C, compare tumor versus wild-type panels; results quantitated in the graph in the bottom right panel). The immunohistochemistry was specific for Y1357 AIB1, since no nuclei were visibly stained in mammary glands from SRC-$3^{-/-}$ (p/CIP$^{-/-}$) mice with the phospho-Y1357 antibody (Fig. 2C, SRC-$3^{-/-}$ panel). Prior incubation of the phospho-Y1357 antibody with a peptide containing the phosphorylated-Y1357 residue (blocking peptide) also resulted in no visible nuclei staining in both wild-type and tumor tissue sections (Fig. 2C, middle panel), further supporting the specificity of the phospho-Y1357 antibody.

Phosphorylation at Y1357 is necessary for AIB1's coactivator function. To help identify functions for phospho-Y1357, a phenylalanine mutant of Y1357 was generated (Y1357F), and its effect on AIB1's ability to function as a transcriptional coactivator was measured using several gene promoter reporters. Our analysis of the role of the Y1357 mutation in these experiments was performed in both full-length AIB1 and a naturally occurring ~130-kDa AIB1-Δ3 isoform which differs from the full-length AIB1 by loss of the first 199 aa. We included the naturally occurring isoform AIB1-Δ3 in addition to the full-length AIB1 in our experiments to define the effect of Y1357F because it has a significantly higher activity on a per mole basis than full-length AIB1 (42, 50). In addition, because of its lower molecular weight, the transfected AIB1-Δ3 isoform can be detected in cell lines, such as COS-7 and HeLa cells, in which the endogenous full-length AIB1 is present at high levels (see Fig. S2 in the supplemental material). Compared to the wild type, the Y1357F mutant had ~50% coactivator activity on the estrogen-responsive promoter reporter in the context of both full-length AIB1 and AIB1-Δ3 (Fig. 3A, left panel). The effect of the Y1357F mutant on AIB1's coactivation ability was also assessed by measuring estrogen-dependent induction of endogenous pS2 mRNA levels in MCF-7 cells. Transient transfection of wild-type AIB1 caused an increase in pS2 message, while no increase was observed with the Y1357 mutant in the presence of estrogen (Fig. 3A, right panel). We also compared the effect of the Y1357F mutant on another hormone-responsive promoter, progesterone-responsive MMTV, and again ob-

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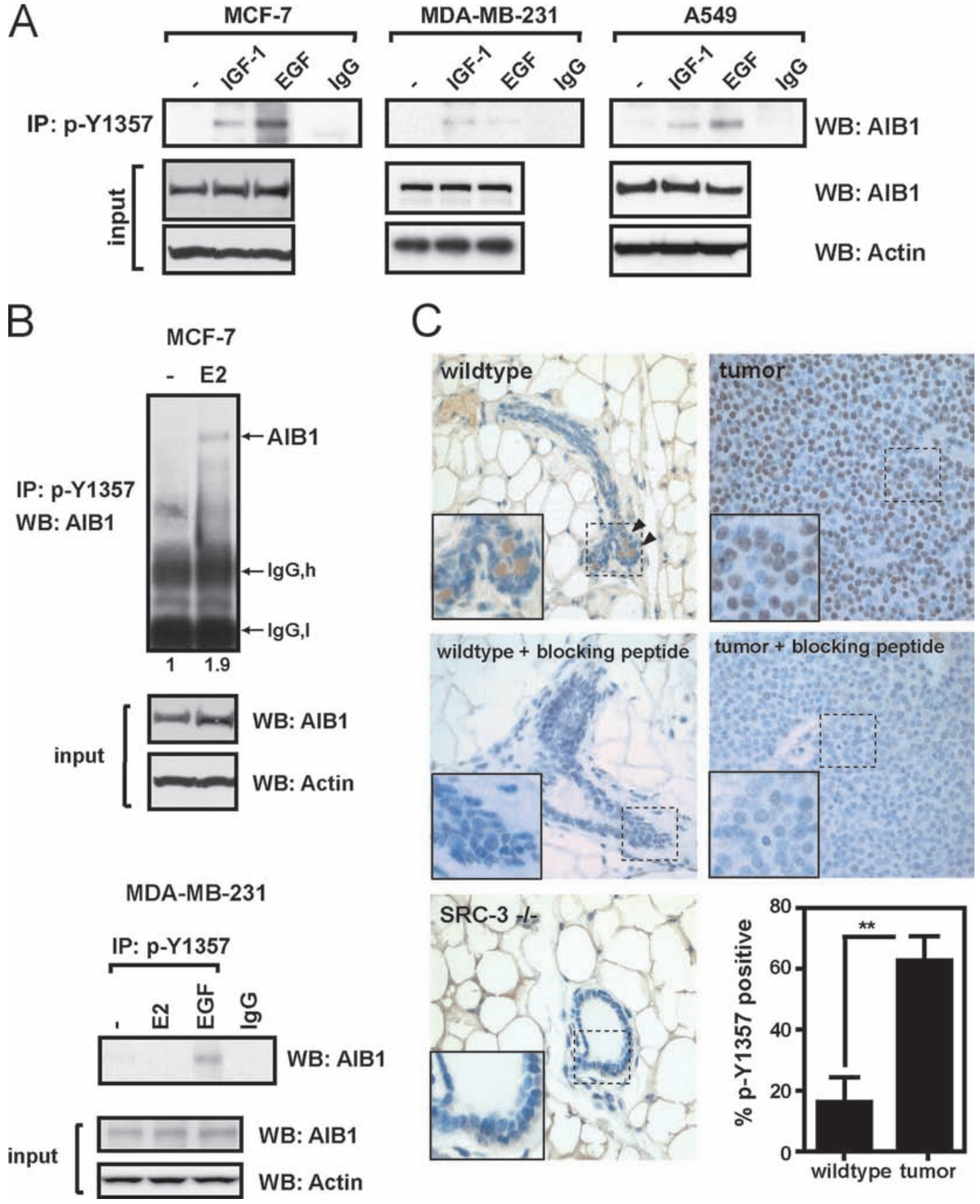
A
MCF-7
MDA-MB-231
A549
- IGF-1 EGF IgG
- IGF-1 EGF IgG
- IGF-1 EGF IgG
IP: p-Y1357
WB: AIB1
input
WB: AIB1
WB: Actin
B
MCF-7
- E2
AIB1
IP: p-Y1357
WB: AIB1
IgG,h
IgG,l
1 1.9
input
WB: AIB1
WB: Actin
MDA-MB-231
IP: p-Y1357
- E2 EGF IgG
WB: AIB1
input
WB: AIB1
WB: Actin
C
wildtype
tumor
wildtype + blocking peptide
tumor + blocking peptide
SRC-3 -/-
% p-Y1357 positive
80
60
40
20
0
**
wildtype tumor

FIG. 2. In vitro and in vivo detection of phospho-Y1357 AIB1. (A) Phospho-Y1357 phosphorylation is observed in breast and lung cancer cell lines following growth factor stimulation using the phospho-Y1357 (p-Y1357) antibody. Cells were treated with 50 ng/ml of IGF or EGF for 10 min or not treated (−). Whole-cell lysates were harvested and used for IP/WB analysis with antibodies as indicated. (B) Estrogen (E2) induced phospho-Y1357 levels in MCF-7 (ER-positive) cells but not in MDA-231 (ER-negative) cells. Hormone-stripped cells were treated for 30 min with either ethanol (−) or E2 (10 nM) before whole-cell lysates were harvested for IP/WB analysis. Heavy (h) and light (l) chains of immunoglobulin G (IgG) are shown to the right of the gel for MCF-7 cells. (C) Increased phospho-Y1357 levels were observed in HER2/neu tumor tissue. Typical IHC staining patterns for phospho-Y1357 expression in paraffin-embedded mammary gland sections from female mice at 11 months (three HER2/neu mice with tumors) and from normal mammary gland 4 from mice at 6 months (three wild-type SRC-3 mice or two SRC-3$^{-/-}$ mice). The phospho-Y1357 blocking peptide and phospho-Y1357 antibody were incubated together on each tissue section for 30 min. A total of 80 to 100 epithelial cells were counted per field. Ten fields were counted per genotype. The graph shows the quantitative results for wild-type and tumor panels. The values in the graph are means plus standard deviations (error bars). The values for phospho-Y1357-positive cells from wild-type mice and mice with tumors were significantly different ($P < 0.0022$) by the unpaired *t* test as indicated by the pair of asterisks.

served that the Y1357F mutation impaired the coactivating functions of AIB1 and AIB1-Δ3 (Fig. 3B).

The effect of the Y1357F mutant on steroid-independent coactivation was tested with multimerized NF-κB and AP-1 promoters. In the context of both AIB1 and AIB1-Δ3, the Y1357F mutant caused a ~40% reduction in the activity of the NF-κB promoter compared to the coactivating effect of wild-type AIB1 and AIB1-Δ3. The reduction in coactivator activity

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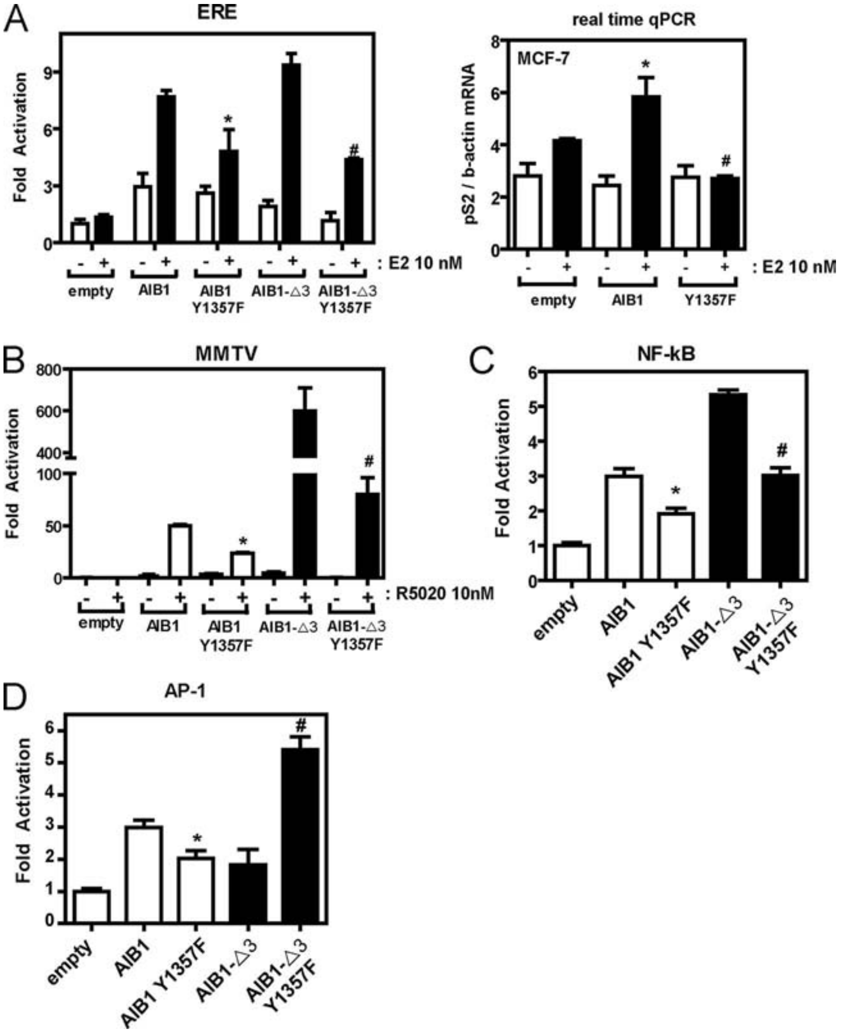

FIG. 3. Functional role for phospho-Y1357 in steroid-dependent and -independent transcription. (A) (Left) Y1357F mutant coactivator effect on estrogen-stimulated transcription. AIB1 and AIB1-Δ3 constructs were cotransfected with ERα and estrogen-responsive promoter reporter (ERE) construct into hormone-stripped COS-7 cells. Cells were treated with ethanol (−) or 10 nM E2 (+) for 24 h and analyzed for reporter activity. Values for the ethanol- and E2-treated cells were significantly different by the unpaired *t* test as indicated by the following symbols: *, $P < 0.03$; #, $P < 0.0012$. (Right) ERα (0.5 μg) and AIB1 (3 μg) constructs were cotransfected into MCF-7 cells for 24 h and treated with E2 for 3 h. Total RNA was harvested and pS2 and beta-actin (b-actin) mRNA levels were measured using real-time quantitative reverse transcription PCR (qPCR). Values for the ethanol- and E2-treated cells were significantly different by the unpaired *t* test as indicated by the following symbols: *, $P < 0.01$; #, $P < 0.001$. (B) Y1357F mutant coactivator activity was measured on a progesterone-dependent promoter. PR-B expression plasmids were cotransfected with the MMTV reporter plasmids into hormone-stripped CHO cells. Cells were treated with either ethanol (−) or 10 nM R5020 (+) for 24 h and then analyzed for reporter activity. Cells from three mice were used for each treatment. Values for the ethanol- and R5020-treated cells were significantly different by two-way analysis of variance as indicated by the following symbols: *, $P < 0.0012$; #, $P < 0.0007$. (C and D) Y1357F mutant's coactivator effects on steroid-independent promoters. HeLa cells were cotransfected with AIB1 expression constructs as indicated and with either a multimerized NF-κB reporter construct (Stratagene Co.) (C) or a multimerized AP-1 reporter construct (Stratagene Co.) (D). Twenty-five nanograms of c-fos and c-jun expression vectors was also cotransfected with the AP-1 reporter. Twenty-four hours after transfection, extracts were prepared for reporter assays. Results are expressed as changes in the level of activation compared with empty vector-transfected cells. Values represent means plus standard deviations (error bars) for quadruplicate wells. Compared to the values for cells transfected with empty vector, the values were significantly different ($P < 0.01$) for the values for cells transfected with AIB1 Y1357F (*) and cells transfected with AIB1-Δ3 Y1357F (#) by the unpaired *t* test.

of the Y1357F mutant on an AP-1 promoter was also observed in the context of full-length AIB1 (Fig. 3D). In contrast, we observed an increase in activity of approximately threefold of the AIB1-Δ3 Y1357F mutant on the AP-1 promoter (Fig. 3C and D), suggesting a role for the N terminus of AIB1 in AP-1-mediated transcription. To investigate the surprising effect of the Y1357F mutation on AP-1-dependent expression further, we analyzed its effect on a promoter fragment from the fibroblast growth factor-binding protein gene (19). The fibroblast growth factor-binding protein promoter is primarily AP-1 dependent and is coactivated by AIB1 in the presence of EGF (42). Although the Y1357F mutant activity was not significantly different than wild-type AIB1-Δ3 in its ability to coactivate this promoter (see Fig. S3 in the supplemental material),

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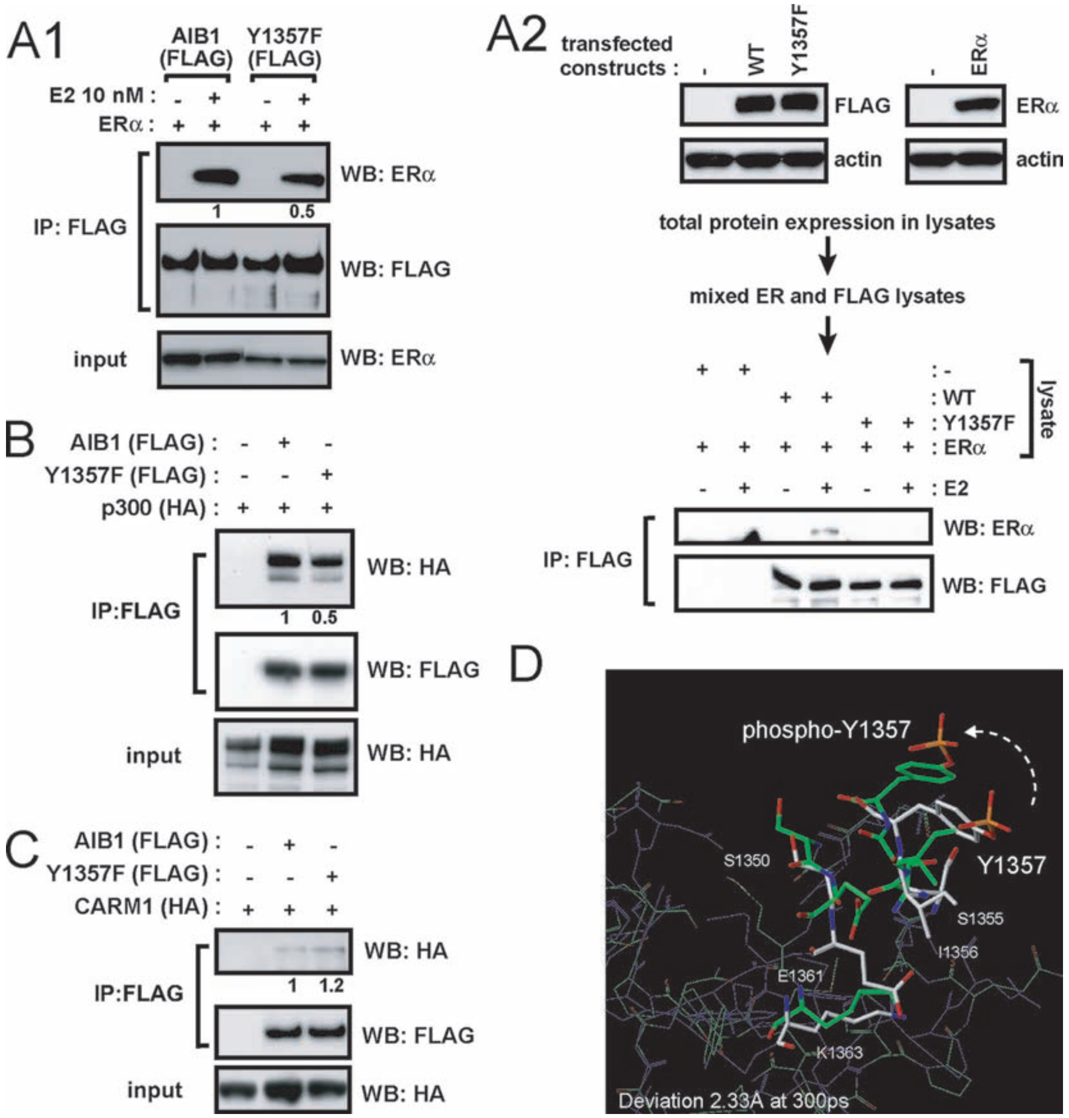
A1
AIB1 (FLAG)
Y1357F (FLAG)
E2 10 nM : - + - +
ERα : + + + +
IP: FLAG
WB: ERα
1
0.5
WB: FLAG
input
WB: ERα
A2
transfected constructs : - WT Y1357F
- ERα
FLAG
ERα
actin
actin
total protein expression in lysates
mixed ER and FLAG lysates
+ + : -
+ + : WT
+ + : Y1357F
+ + + + + + : ERα
lysate
- + - + - + : E2
IP: FLAG
WB: ERα
WB: FLAG
B
AIB1 (FLAG) : - + -
Y1357F (FLAG) : - - +
p300 (HA) : + + +
IP:FLAG
WB: HA
1 0.5
WB: FLAG
input
WB: HA
C
AIB1 (FLAG) : - + -
Y1357F (FLAG) : - - +
CARM1 (HA) : + + +
IP:FLAG
WB: HA
1 1.2
WB: FLAG
input
WB: HA
D
phospho-Y1357
S1350
Y1357
S1355
I1356
E1361
K1363
Deviation 2.33A at 300ps

FIG. 4. Phosphorylation of Y1357 modulates transcription cofactor interactions. Interaction of the AIB1 Y1357F mutant with transcription cofactors ERα (A1 and A2), p300-HA (B), and CARM1-HA (C). ERα, p300-HA, and CARM1-HA expression plasmids were separately cotransfected (+) with AIB1-Δ3-FLAG [AIB1 (FLAG)] constructs in 293T cells, and whole-cell lysates were prepared 24 h later for IP/WB analysis. The ratio of the amount of nonmutated AIB1-FLAG immunoprecipitated with the target protein (ERα, p300, or CARM1) was standardized to 1 and compared with the ratio of Y1357F FLAG immunoprecipitated with the target protein. These ratios are indicated below the panels. In panel A1, 293T cells were treated with ethanol (−) or 10 nM E2 (+) for 1 h before whole-cell lysates were prepared for analysis. The ERα input panel represents noncontiguous lanes of the same WB. In panel A2, empty vector (−), ERα, and AIB1 constructs were transfected separately into 293T cells and lysates were prepared. ERα- and AIB1-containing lysates were mixed and treated with either ethanol (−) or 100 nM E2 (+) before immunoprecipitation was performed. (D) Simulated effect of phosphorylated Y1357 on the local structure of AIB1. Amino acids with a white backbone were not phosphorylated, while amino acids with a green backbone were phosphorylated.

there was a trend toward increased activity even in the presence of a single AP-1 element in this promoter. The altered function of the Y1357F mutant's ability to coactivate both hormone- and growth factor-responsive promoters was not due to differences in exogenous AIB1 expressed protein levels (see Fig. S2 in the supplemental material). Overall, these functional data indicate that phosphorylation at Y1357 in AIB1 is important for both steroid-dependent and -independent transcriptional control, although the impact of Y1357 phosphorylation is highly dependent on the promoter context.

Phosphorylation of Y1357 alters AIB1 interaction with transcription cofactors. Since the phosphorylation status of Y1357 affected AIB1's coactivating ability on steroid- and NF-κB-dependent promoters, we postulated that phosphorylation can affect functional interactions between AIB1 and other proteins assembled in transcription complexes formed in response to steroid hormones and growth factor signals. We first examined interactions with the estrogen receptor ERα. In IP assays, we found ~50% less interaction between the Y1357F-FLAG mutant and ERα (Fig. 4A1). However, when AIB1 and ERα were

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cotransfected together, we consistently observed a slight reduction in total ERα levels when cotransfected with Y1357F mutant. To determine whether the interaction between ERα and Y1357F was reduced due to an alteration in their binding affinity and not due to a reduction in total ERα available for interaction, we transfected the FLAG-tagged AIB1 and ERα constructs separately into 293T cells and mixed the lysates in the presence or absence of estrogen and then performed the FLAG IP followed by Western blotting for ERα (Fig. 4A2). Total expression of levels of AIB1 and ERα were also evaluated in the input lysates. With equal expression of ERα and AIB1, we observed a marked decrease in the affinity of Y1357F mutant for ERα compared to wild-type AIB1 (Fig. 4A2).

The interaction of AIB1 with CBP/p300, a histone acetyltransferase, is also a critical interaction for coactivation (8). HA-tagged p300 was cotransfected with FLAG-tagged AIB1 or Y1357F mutant construct, and coimmunoprecipitations were performed with the anti-FLAG antibody. Again, the Y1357F mutant interacted ~50% less than AIB1 in this assay for binding to p300 (Fig. 4B). We also noticed that Y1357 is close to a CARM1 methylation and interaction site on AIB1 (Fig. 1C). Unlike CBP/p300, engagement of the CARM1 cofactor has been demonstrated to inhibit transcription complex formation and to have a repressive effect on gene transcription (33). In contrast to the interaction results with p300 and ERα, we observed slightly increased amounts of CARM1 binding to the Y1357F mutant (Fig. 4C) compared to nonmutated AIB1. These results suggest that phosphorylation of this residue may play a minor role in stabilizing the interaction of AIB1 with CARM1. Overall, these data support the role of Y1357 phosphorylation in controlling the interaction between AIB1 and cofactors, such as ERα, p300, and CARM1, that ultimately alter its transcriptional activity.

Since mutation of Y1357 altered interactions with ERα and p300, we investigated whether Y1357 phosphorylation caused discernible differences in AIB1's structure that could explain changes in cofactor binding. Protein structure predictions were made with the MODELLER 7v7 program and 300-ps molecular dynamics simulations of the region surrounding Y1357 and phospho-Y1357 were carried out using distance-dependent dielectric constants (Fig. 4D). Upon phosphorylation, both phospho-Y1357 and nearby residues S1350, S1355, I1356, and E1361 (amino acids with a green backbone) move away from one another to avoid steric hindrance with the added, charged phosphate group, illustrating possible structural and functional roles for both Y1357 and phospho-Y1357. Therefore, phosphorylation at Y1357 could cause local structural alterations that increase the stability of AIB1's interactions with transcription machinery components, such as p300 and ERα, while dephosphorylation could maintain the stability of CARM1 binding, at the expense of p300 and ERα binding.

AIB1 Y1357 is phosphorylated by the Abl kinase pathway. Since we determined that phosphorylation at Y1357 had a functional role for AIB1's ability to coactivate by promoting the formation of transcription cofactor complexes, we wanted to determine the tyrosine kinase that was responsible for Y1357 phosphorylation. To narrow down the possible tyrosine kinases that could phosphorylate AIB1, the amino acid sequences around Y1357 were analyzed using Scansite 2.0 software program to determine whether the sequences formed a consensus substrate for a particular tyrosine kinase (36). The Scansite program predicted that Y1357 and surrounding residues in AIB1 was a possible Abl tyrosine kinase substrate based on the presence of isoleucine at position −1 to Y residue which was also found in other substrates of Abl kinase, such as Dok (60), and Cas (44) (Fig. 5A). A general consensus for Abl kinase phosphorylation substrate has been derived from six known substrates (4, 10, 12, 44, 60, 63) (Fig. 5A). Interestingly, the isoleucine at position −1 was a given a higher selectivity value than the proline at position +3 in a study that originally characterized Abl's substrate sequence specificity (47). However, it appears from the comparison in Fig. 5A that the proline at position +3 is a common feature of many known Abl substrates. To determine whether AIB1 was indeed phosphorylated by Abl kinase, we first performed an in vitro kinase assay to determine whether a GST fragment containing the Y1357 residue could be phosphorylated by recombinant Abl kinase. We found that a GST-AIB1 fragment from 1017 to 1420 aa was readily phosphorylated at Y1357 by exogenous Abl kinase, as detected by the phospho-Y1357 antibody (Fig. 5B). To confirm that Abl kinase could phosphorylate AIB1 in whole cells, we overexpressed Abl kinase using an Abl-AU5-tagged construct and cotransfected it with an AIB1-FLAG construct into 293T cells. We immunoprecipitated AIB1 with either a FLAG or phospho-Y1357 antibody and detected phosphorylated AIB1 by Western blotting. Since CrkL is an Abl/Bcr-Abl substrate (11), phospho-CrkL (Y207) levels were measured (Fig. 5C, input panels) to ensure that the transfected Abl kinase was functional. Consistent with the in vitro kinase assay, we detected a large amount of Y1357 phosphorylation only in the presence of transfected active Abl kinase (Fig. 5C, IP: FLAG panels).

Abl has the ability to phosphorylate and bind directly to its substrate targets, such as c-Jun (4) and Cas (44). We therefore determined whether Abl has the ability to complex with AIB1 and whether this binding was affected by the phospho-Y1357 residue. We cotransfected Abl with either AIB1 or the AIB1 Y1357F mutant into 293T cells and examined their interaction with Abl kinase by coimmunoprecipitation and WB analysis. Abl interacted strongly with AIB1, and ~50% of this binding was lost between Abl and the Y1357F mutant (Fig. 5D, IP: FLAG, WB: AU5 gel, lane 3). This result indicated that phosphorylation of the Y1357 residue increased the affinity for Abl kinase but was not absolutely required for the AIB1 interaction with Abl kinase. Like other nonreceptor tyrosine kinases, Abl mainly exists intracellularly in an inactive form and becomes activated by either external signals, such as growth factor stimulation or cell adhesion, or as a response to DNA damage (as reviewed in reference 39). Conversely, the Abl kinase inhibitor imatinib (Gleevec; STI-571) binds to the ATP binding pocket when Abl is in its inactive conformation (45). To determine whether the activation of Abl kinase was necessary for the interaction with AIB1, 293T cells were pretreated with imatinib 1 h prior to harvesting the cells for IP analysis. Inhibition of Abl kinase activity eliminated phosphorylation at Y1357 and completely prevented the interaction between Abl and AIB1 (Fig. 5D, IP: FLAG, WB: AU5 gel, lane 4). This result strongly suggests that AIB1 can interact only with the active form of Abl kinase. The inhibition of Abl kinase activity by imatinib was confirmed by measuring phospho-CrkL levels (Fig. 5D,

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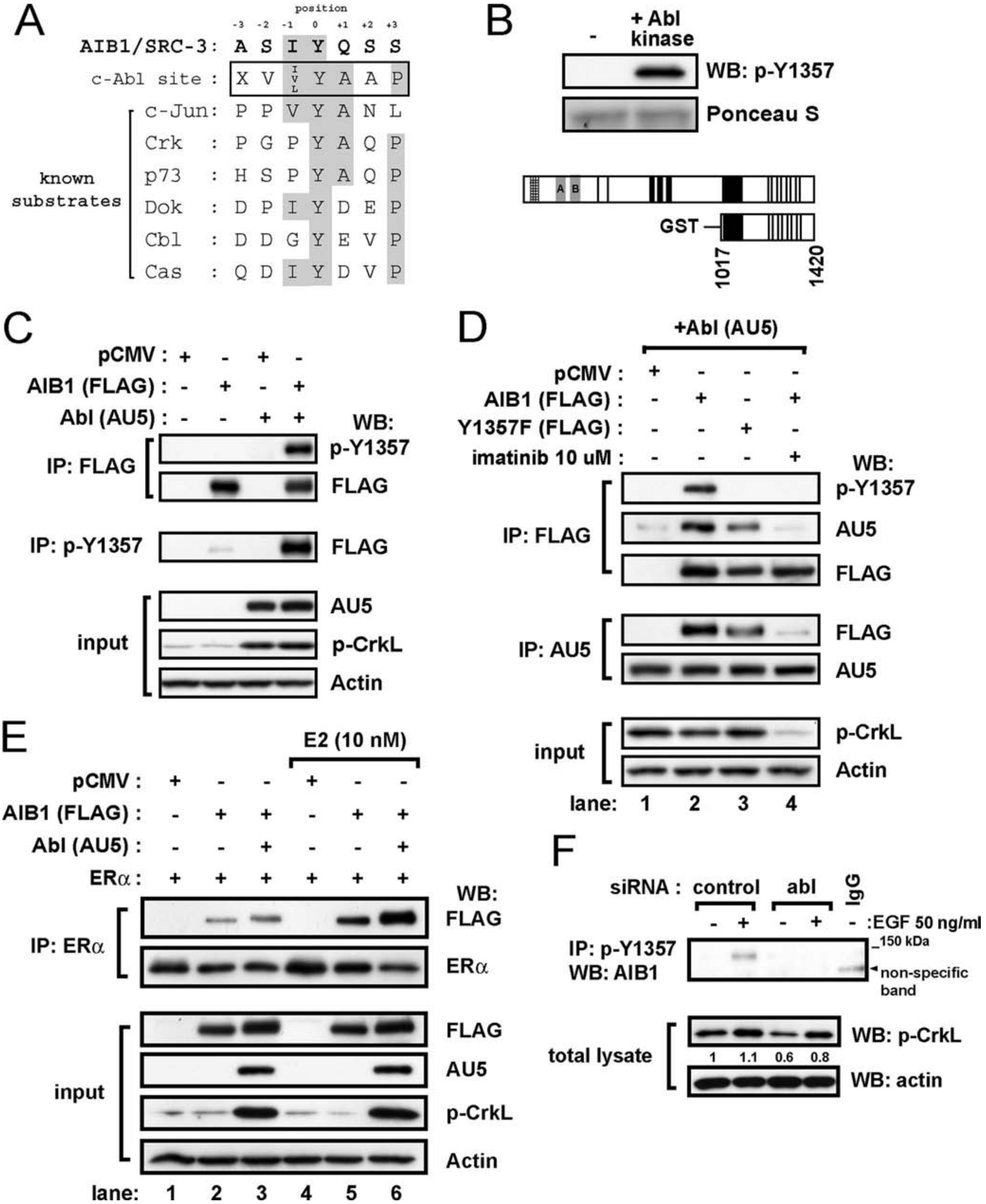

FIG. 5. Abl kinase directly phosphorylates phospho-Y1357 and binds to AIB1. (A) AIB1 Y1357 contains a partial Abl kinase recognition site. Amino acids immediately surrounding the AIB1 Y1357 residue were compared to an Abl kinase consensus sequence peptide and known Abl kinase substrates. Amino acids that are identically positioned are highlighted. (B) Abl kinase phosphorylates an AIB1 GST fragment in vitro. An in vitro kinase assay was performed with purified GST-AIB1 protein (aa 1017 to 1420) and recombinant Abl kinase. (C) Expression of constitutively active Abl phosphorylates AIB1 at Y1357. Abl-AU5 was cotransfected with AIB1-Δ3-FLAG constructs in 293T cells. Phosphorylated

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input, lane 4). Since we observed that estrogen could increase the phosphorylation of the Y1357 site (Fig. 2B) and that conversely, mutation of the Y1357 site diminished ERα interaction with AIB1 interaction (Fig. 4A), we were also interested to determine how increasing Abl kinase activity would affect the ERα-AIB1 complex formation. To accomplish this, we transfected 293T cells with a combination of ERα, Abl-AU5, and AIB1-FLAG expression constructs and determined by immunoprecipitation and Western blot analysis the amount of ERα-AIB1 complex formation in the presence or absence of added estrogen. As expected, IP of ERα brings down AIB1, and this interaction is increased in the presence of 10 nM estrogen (Fig. 5E, lane 5). Some interaction with ERα and AIB1 was observed in the absence of estrogen. Due to the high expression of transfected ERα, residual estrogens in the charcoal-stripped serum media was enough to cause some ERα-AIB1 complex formation (Fig. 5E, lanes 2 and 3). Interestingly, when Abl kinase is active, a significant increase in the amount of complex between ERα and AIB1 occurs (Fig. 5E, lane 6). Consistent with the idea that Abl phosphorylates AIB1, we also observed a significant upward mobility shift in the immunoprecipitated AIB1 in the lanes where Abl kinase is overexpressed (Fig. 5E, lanes 3 and 6). These data suggest that phosphorylation of AIB1 by Abl kinase facilitates the interaction with ERα and this is considerably enhanced in the presence of estrogen. To confirm that Abl kinase phosphorylates AIB1 in a breast cancer cell line, we used an siRNA directed against endogenous Abl kinase to determine whether reducing Abl kinase levels/activities resulted in a corresponding decrease in phospho-Y1357 levels. As shown in Fig. 2A, EGF treatment in MDA-231 cells resulted in an increase in phospho-Y1357 levels. When Abl kinase activity was reduced in MDA-231 cells with siRNA transfection, phospho-Y1357 levels were reduced dramatically (Fig. 5F). Total levels of Abl were difficult to detect in MDA-231 cells; therefore, phospho-CrkL activation was used as a surrogate marker for Abl siRNA knockdown. We observed a 20 to 40% decrease in phospho-CrkL levels when transfected with the Abl siRNA (Fig. 5F). These data clearly indicate that the Y1357 site on AIB1 is a substrate for Abl kinase in breast cancer cells.

Abl activity and phospho-Y1357 site contribute to AIB1's function as a critical coactivator and role in tumorigenesis. To assess the effect of Abl on AIB1 coactivator activity in MCF-7 cells, we inhibited endogenous Abl in MCF-7 cells with imatinib. MCF-7 cells carry the AIB1 gene amplification and therefore express very large amounts of AIB1 protein. Imatinib inhibited both basal and exogenous AIB1 coactivation of a MMTV promoter reporter in the presence of R5020 (Fig. 6A). AIB1 is rate limiting for estrogen-induced growth of MCF-7 cells (28). Imatinib or Abl siRNA treatment significantly reduced MCF-7 cell growth after 4 days of estrogen treatment (Fig. 6B). These findings demonstrate that Abl activity is necessary for AIB1's coactivation of hormone-dependent gene promoters and, ultimately, necessary for hormone-dependent growth of breast cancer cells. To directly assess the Y1357 site's contribution to AIB1-dependent tumorigenesis, focus formation assays were performed with transiently transfected H-ras V12 and the Y1357F mutant constructs in AIB1/SRC-$3^{-/-}$ MEFs. AIB1 has been shown to reduce the incidence and latency of breast tumors in the MMTV v-Ha-ras mammary tumorigenesis mouse model (24). Wild-type AIB1 alone or the Y1357F mutant did not induce focus formation (data not shown), while H-ras V12 alone did result in the formation of a limited number of foci. Wild-type AIB1 plus H-ras V12 produced an increased number of foci, while the Y1357F mutant plus H-ras V12 produced fewer foci (Fig. 6C, chart). These data demonstrate that the Y1357 site directly contributes to AIB1's role in an oncogene-dependent transformation assay. We propose a molecular model (Fig. 6D) in which activated Abl binds to and phosphorylates AIB1 at Y1357. Phosphorylated-Y1357 AIB1 leads to a conformational alteration that stabilizes AIB1's interaction with cofactors, such as ERα and p300, while simultaneously resulting in a less stable interaction with CARM1. Phosphorylation at Y1357 is required for AIB1's ability to mediate steroid receptor-dependent gene transcription as well as its ability to contribute to breast cancer tumorigenesis.

DISCUSSION

This is the first study, to our knowledge, that describes the tyrosine phosphorylation of a steroid receptor coactivator. Although AIB1 tyrosine phosphorylation is initiated by membrane tyrosine kinases, it appears to be eventually mediated by Abl, a nonreceptor tyrosine kinase. Our results are consistent with a model outlined in Fig. 6D whereby Abl kinase is activated by an extracellular signal and in its activated form creates a complex with AIB1. AIB1 is then rapidly phosphorylated by Abl at tyrosine Y1357, thereby changing its local conformation and increasing its affinity for p300 and steroid receptors and decreasing its affinity for the repressor CARM1. At promoters that harbor estrogen, progesterone, or NF-κB response elements, this leads to an overall increase in transcription. At other promoter elements, such as AP-1 sites, the tyrosine phosphorylation of AIB1 seems to be less important in formation of the transcription complex and may normally even repress transcription. This suggests that other AIB1 cofactor interactions may play a rate-limiting role in this promoter context. Inter-

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CrkL (P-CrkL) (Y207) levels were detected to determine Abl activation. (D) Interaction between Abl and AIB1 is partially mediated by Y1357 and is fully dependent on Abl kinase activity. Abl-AU5 and AIB1-Δ3-FLAG (AIB1 or Y1357F) constructs were used as described above for panel C. Transfected 293T cells were pretreated for 4 h with either dimethyl sulfoxide (−) or 10 μM imatinib prior to collection of lysates and IP. (E) Abl forms a complex with ERα and AIB1 in the presence (+) of estrogen. 293T cells were transfected (+) with Abl-AU5, ERα, and AIB1-Δ3-FLAG for 24 h and treated with either ethanol (lanes 1 to 3) or 10 nM E2 before whole-cell lysates were harvested. Lysates were immunoprecipitated with ERα followed by Western blot analysis for FLAG or ERα. (F) Reduction of Abl results in a decrease in endogenous AIB1 Y1357 phosphorylation in MDA-231 cells. MDA-231 cells were transfected with Abl (exon 11) siRNA for 48 h, serum starved, and treated with vehicle (−) or EGF (+) for 10 min. Phosphorylated CrkL (P-CrkL) levels were used to assess reduction in Abl activity. Activated CrkL levels were quantitated as ratios of the control siRNA-untreated lane. IgG, immunoglobulin G.

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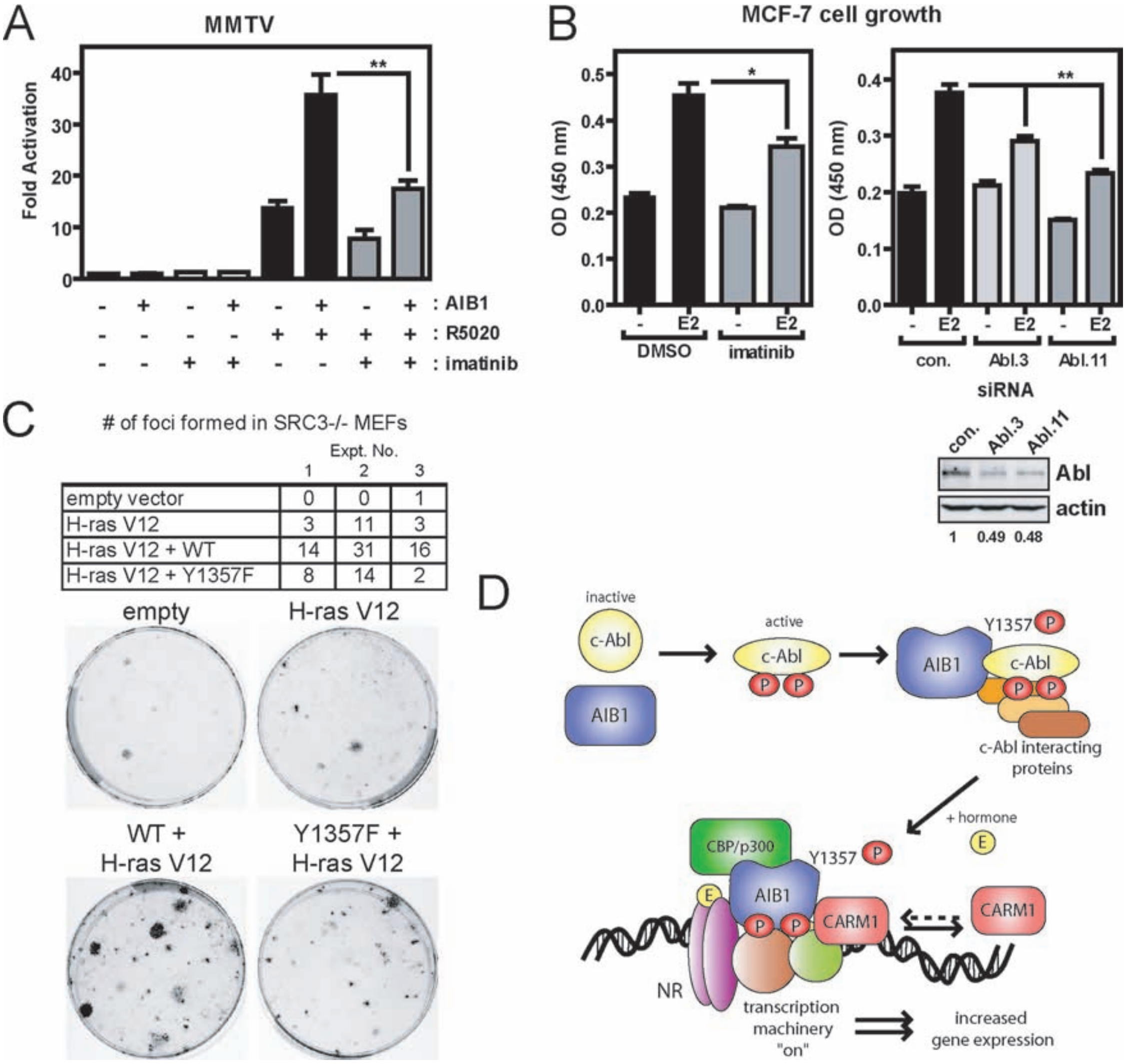

# of foci formed in SRC3-/- MEFs

	Expt. No. 1	2	3
empty vector	0	0	1
H-ras V12	3	11	3
H-ras V12 + WT	14	31	16
H-ras V12 + Y1357F	8	14	2

FIG. 6. Abl activity is necessary for AIB1's role in hormone-induced promoter coactivation and proliferation of breast cancer cells. (A) Inhibition of Abl kinase activity by imatinib reduces AIB1's ability to coactivate progesterone-dependent gene promoter activity. MCF-7 cells were transfected with MMTV reporter, PR-B, and AIB1 vectors for 24 h, pretreated 1 h with 10 μM imatinib, and then treated with 10 nM R5020 with 10 μM imatinib for an additional 24 h before reporter analysis. Each experiment was performed in triplicate. Results are expressed as changes in the activation of cells transfected with an empty vector. Values that were significantly different ($P < 0.002$) by two-way analysis of variance are indicated by the bracket and pair of asterisks. (B) Inhibition of Abl kinase significantly reduces E2-induced cell growth of MCF-7 breast cancer cells. For imatinib growth assays, hormone-stripped MCF-7 cells were pretreated with 10 μM imatinib for 1 h and then treated with ethanol or 10 nM E2 with imatinib for 4 days. For Abl siRNA growth assays, hormone-stripped MCF-7 cells were transfected with scrambled (control [con.]) siRNA or with abl.3 or abl.11 siRNAs (specific for exon 3 or 11) for 24 h. Cells were treated with ethanol (−) or 10 nM E2 for 4 days. Each experiment was performed in triplicate. Values that were significantly different by two-way analysis of variance are indicated by brackets and the following symbols: *, $P < 0.001$; **, $P < 0.0002$. In panels A and B, values are means plus standard deviations (error bars). (C) Y1357F mutant demonstrates reduced H-ras V12-dependent focus formation in AIB1/SRC-3$^{-/-}$ MEFs. AIB1/SRC-3$^{-/-}$ MEFs was transfected with H-ras V12 and empty vector, AIB1-Δ3 (wild type [WT]) or AIB1-Δ3 Y1357F (Y1357F) constructs. After 3 weeks, focus formation was assessed after staining with crystal violet. Three independent experiments were performed. (D) A proposed model for the role of Abl tyrosine phosphorylation of AIB1 in steroid receptor signaling. Activated Abl binds to and phosphorylates AIB1 at Y1357. Phospho-Y1357 AIB1 stabilizes its interaction with cofactors, such as ERα and p300, while simultaneously resulting in a less stable interaction with CARM1. P, phosphate group; E, estrogen; NR, nuclear receptor.

estingly, it has been shown that AP-1-mediated transcription is impacted by serine and threonine phosphorylation of AIB1 (57). Furthermore, it has been postulated that phosphorylation at a particular residue of AIB1 may be a driving event, enabling subsequent posttranslational modifications (55). It would of interest to determine whether Y1357 is a primary permissive phosphorylation or a secondary occurrence after other posttranslational modifications including as yet uncharacterized additional tyrosine, serine, and threonine phosphorylation sites in AIB1. Tyrosine phosphorylation is usually a consequence of rapid activation of growth factor receptor tyrosine kinases and cytoplasmic protein tyrosine kinases upon ligand stimulation. Therefore, it may be more likely that tyrosine phosphorylation of AIB1 is an early rate-limiting modification which influences

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phosphorylation or posttranslational modifications at other sites.

The phosphorylation of AIB1 by Abl kinase was a somewhat surprising result, especially as the Abl kinase consensus surrounding the Y1357 residue is not highly conserved. The role of Abl kinase in oncogenesis is complex. The oncogenic forms for Abl, v-Abl and Bcr-Abl, have been extensively studied and well described; however, the normal cellular functions of Abl are still being characterized (39, 52). Unlike Src tyrosine kinase, Abl has been found to have both a cytoplasmic and nuclear function, and it has profoundly different functions depending on is subcellular localization. Cytoplasmic Abl is associated with cell growth, motility, migration, and adhesion, while nuclear Abl is associated with apoptosis (15, 52). Similar to Abl kinases, AIB1 is also both a cytoplasmic and nuclear protein, albeit the full-length protein appears to be predominantly nuclear (29, 41). The mechanisms which alter the localization of AIB1 have been a topic of intense focus, as it may be important in regulating posttranslational modifications and protein stability of AIB1 (2, 26, 62). It would be of interest to determine whether the Abl-AIB1 interaction and phosphorylation occurs in a specific subcellular compartment, what other modifications precede or follow Y1357 phosphorylation, and the resulting functional consequences.

Our results strongly suggest that phosphorylation of and interaction with AIB1 by Abl kinase play a role in either Abl- or AIB1-mediated oncogenesis. As stated above, Abl can have different roles in oncogenesis depending on its subcellular localization and also the level of its activated expression. Similarly, AIB1 can be oncogenic when overexpressed in mammary epithelium and other epithelial tissue (50, 51, 61). Conversely, AIB1/SRC-3$^{-/-}$ transgenic mice develop lymphomas as they age (9), suggesting that in this context AIB1 may normally suppress oncogenesis. It would be of interest to determine whether different functional interactions between Abl and AIB1 in the hematopoietic system compared with epithelial cells alter the role of AIB1 in oncogenesis. It may be possible that an epithelial tissue growth factor and steroid receptor pathways activate Abl and thus AIB1. However, in the hematopoietic system, a different paradigm may operate between Abl and AIB1, possibly in a different subcellular compartment. These are intriguing questions for further study.

Abl is activated by platelet-derived growth factor (PDGF) and EGF (40), but whether IGF-1 or insulin is a possible activator of Abl kinase seems to be somewhat cell line dependent and is still not fully understood (14, 46, 48). Regardless of the extracellular activator of Abl kinase, we postulate that other intracellular Abl-activated proteins (Fig. 6D) will be a necessary part of the Abl-AIB1 complex. Abl usually exists in an inhibited state in which either Abl keeps both its kinase domain and Src homology 2 (SH2)/SH3 domain tightly bound to itself (18, 35) or by binding to inhibitory proteins, such as ABI-1 (39). Activation of Abl kinase, perhaps due to phosphorylation (6, 34), results in exposure of the N-terminal myristoyl group and exposure of the SH2/SH3 domains to bind to phosphotyrosine proteins. It has been postulated that Abl substrates are initially phosphorylated by basal kinase activity of Abl, which initiates a positive-feedback loop by activating SH2 domain-dependent activation of Abl and finally results in the recruitment of its substrate (18). Discovering the components of the AIB1-Abl kinase complex, especially a SH2/SH3 domain-containing protein that also binds to AIB1, may add further levels of complexity to the regulation of AIB1 function.

A possible clinical application of this study is the utilization of the phospho-specific antibody to detect phosphorylated AIB1 at Y1357 as a marker for activated Abl kinase in tumors and possible responsiveness to Abl kinase inhibitors, such as imatinib. At the writing of this article, seven clinical trials were ongoing to study the beneficial effects of using imatinib in conjunction with other therapies to treat metastatic breast cancer. One of the inclusion criteria of these trials is the presence of molecular markers, c-kit and PDGF receptor (PDGFR) beta. Autocrine PDGF/PDGFR signaling has been shown to promote metastasis in MMTV-Neu transgenic mice, and imatinib treatment was shown to reduce metastasis (21). This finding is interesting, since we also observed an increase in activated phospho-Y1357 AIB1 in HER2/neu tumors (Fig. 2C), thus suggesting that AIB1 may be downstream of PDGFR signaling. It will be interesting to determine in patient samples the levels of tyrosine-phosphorylated AIB1 and whether this is predictive of outcome in therapies directed at reducing growth factor and/or Abl kinase signaling. Since Abl kinase promotes complex formation between ERα and AIB1, as well as reducing NFκB-mediated transcription, imatinib may have an inhibitory effect on mammary tumor growth in both steroid-dependent and -independent settings in breast cancer. Finally, due to the successful use of imatinib in the treatment of multiple human leukemias and the emergence of imatinib resistance in patients, a large number of drugs that target Abl, PDGFR beta, and Src are in the pipeline for drug development and testing. These inhibitors may also be applicable in the treatment of breast cancer, especially those that have high levels of phospho-Y1357 AIB1.

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Research article

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Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis

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Abstract

Introduction Estrogen deprivation using aromatase inhibitors is one of the standard treatments for postmenopausal women with estrogen receptor (ER)-positive breast cancer. However, one of the consequences of prolonged estrogen suppression is acquired drug resistance. Our group is interested in studying antihormone resistance and has previously reported the development of an estrogen deprived human breast cancer cell line, MCF-7:5C, which undergoes apoptosis in the presence of estradiol. In contrast, another estrogen deprived cell line, MCF-7:2A, appears to have elevated levels of glutathione (GSH) and is resistant to estradiol-induced apoptosis. In the present study, we evaluated whether buthionine sulfoximine (BSO), a potent inhibitor of glutathione (GSH) synthesis, is capable of sensitizing antihormone resistant MCF-7:2A cells to estradiol-induced apoptosis.

Methods Estrogen deprived MCF-7:2A cells were treated with 1 nM 17β-estradiol (E_2), 100 μM BSO, or 1 nM E_2 + 100 μM BSO combination *in vitro*, and the effects of these agents on cell growth and apoptosis were evaluated by DNA quantitation assay and annexin V and terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) staining. The in vitro results of the MCF-7:2A cell line were further confirmed *in vivo* in a mouse xenograft model.

Results Exposure of MCF-7:2A cells to 1 nM E_2 plus 100 μM BSO combination for 48 to 96 h produced a sevenfold increase in apoptosis whereas the individual treatments had no significant effect on growth. Induction of apoptosis by the combination treatment of E_2 plus BSO was evidenced by changes in Bcl-2 and Bax expression. The combination treatment also markedly increased phosphorylated c-Jun N-terminal kinase (JNK) levels in MCF-7:2A cells and blockade of the JNK pathway attenuated the apoptotic effect of E_2 plus BSO. Our *in vitro* findings corroborated *in vivo* data from a mouse xenograft model in which daily administration of BSO either as a single agent or in combination with E_2 significantly reduced tumor growth of MCF-7:2A cells.

Conclusions Our data indicates that GSH participates in retarding apoptosis in antihormone-resistant human breast cancer cells and that depletion of this molecule by BSO may be critical in predisposing resistant cells to E_2-induced apoptotic cell death. We suggest that these data may form the basis of improving therapeutic strategies for the treatment of antihormone resistant ER-positive breast cancer.

Introduction

Currently, estrogen deprivation using aromatase inhibitors is one of the standard treatments for postmenopausal women with estrogen receptor (ER)-positive breast cancer [1]. Unfortunately, a major clinical problem with the use of prolonged estrogen deprivation is the development of drug resistance (that is, hormone-independent growth) [2,3]. Our laboratory as well as other investigators, have instigated a major effort in

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BSO: L-buthionine sulfoximine; E_2: 17β-estradiol; ER: estrogen receptor; FBS: fetal bovine serum; GCS: glutamylcysteine; GPx2: glutathione peroxidase; GS: glutathione synthetase; GSH: glutathione; H&E: hematoxylin and eosin; JNK: c-Jun N-terminal kinase; Rh123: rhodamine 123; SFS: dextran coated charcoal-treated FBS; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.

studying antihormone resistance in breast cancer and have developed model systems of estrogen deprivation that are sensitive [4-6] or resistant to the apoptotic actions of estrogen [7]. In particular, we have previously reported the development of an estrogen deprived breast cancer cell line, MCF-7:5C, which undergoes estradiol-induced apoptosis after 2 days of treatment via the mitochondrial pathway [8]. In contrast, we have another estrogen deprived breast cancer cell line, MCF-7:2A, which appears to be resistant to estradiol-induced apoptosis [7]. We are studying resistance to estrogen induced apoptosis because clinical experience shows us that only 30% of patients respond to estrogen induced apoptosis once exhaustive antihormonal therapy occurs [9]. An important goal would be to see whether the apoptotic effect of estrogen can be enhanced in antihormone resistant cells. This new, targeted approach to the treatment of metastatic breast cancer could open the door to novel approaches to treatment with drug combinations.

L-Buthionine sulfoximine (BSO) is a specific γ-glutamylcysteine synthetase inhibitor that blocks the rate-limiting step of glutathionine (GSH) biosynthesis and in doing so depletes the intracellular GSH pool in both cultured cells and in whole animals [10]. GSH is a water-soluble tripeptide composed of glutamine, cysteine, and glycine. Reduced glutathione is the most abundant intracellular small molecule thiol present in mammalian cells and it serves as a potent intracellular antioxidant protecting cells from toxins such free radicals [11,12]. Changes in GSH homeostasis have been implicated in the etiology and progression of a variety of human diseases, including breast cancer [13]. In particular, studies have shown that elevated levels of GSH prevent apoptotic cell death whereas depletion of GSH facilitates apoptosis [10,14]. BSO depletes cellular GSH [10] and sensitizes tumor cells to apoptosis induced by standard chemotherapeutic agents [15,16].

Apoptosis (programmed cell death) is required for normal development and tissue homeostasis in multicellular organisms. Deregulation of apoptosis is fundamental to many diseases, such as cancer, stroke, heart disease, neurodegenerative disorders, and autoimmune disorders [17]. There are two main pathways for apoptosis, namely the extrinsic receptor mediated pathway and the intrinsic mitochondria-mediated pathway [18,19]. Components of the extrinsic pathway include the death receptors FasR/FasL, DR4/DR5, and tumor necrosis factor (TNF) [20], whereas the intrinsic pathway centers on the Bcl-2 family of proteins which comprises both proapoptotic proteins, such as Bax, Bak, and Bid and antiapoptotic proteins, such as Bcl-2 and Bcl-xL [18,19]. The Bcl-2 family proteins regulate apoptosis by altering mitochondrial membrane permeabilization which leads to the release of apoptogenic factors such as cytochrome *c*, procaspases, and apoptosis inducing factor (AIF). In particular, Bcl-2 and Bcl-xL inhibit apoptosis by maintaining mitochondrial membrane integrity whereas Bax and Bak facilitate apoptosis by initiating the loss of outer mitochondrial integrity [21]. Apart from its action on the mitochondria, there is also evidence that Bcl-2 possesses antioxidant property. Bcl-2 overexpression increases cellular GSH level which is associated with increased resistance to chemotherapy-induced apoptosis [22,23] whereas GSH depletion restores apoptosis in Bcl-2 expressing cells [16].

Based on microarray studies we found that the antihormone resistant MCF-7:2A cells express markedly elevated levels of glutathione synthetase (GS) and glutathione peroxidase 2 (GPx2); two enzymes that are involved in glutathione synthesis, which suggests that resistance to estrogen-induced apoptosis might be due to elevated levels of GSH present in the cells. If MCF-7:2A cells do indeed possess high levels of GSH, then it is possible that the use of BSO – as a single agent – might be able to sensitize these cells to estrogen-induced apoptosis. As mentioned before, there is current clinical interest in using low dose estradiol therapy to treat antihormone resistant breast cancer [24] however only a minimal 30% of patients respond to this therapeutic strategy. A combination of BSO and estradiol could possibly be used to improve the efficacy of estradiol as an apoptotic agent if glutathione depletion is fundamental to tumor cell survival. We have addressed the hypothesis that by altering glutathione levels we may be able to enhance apoptosis to estrogen and have employed BSO as our agent of choice because of earlier work clinically, which may provide a foundation for subsequent clinical trials.

In the present study, we show that depletion of cellular GSH by BSO sensitizes antihormone-resistant MCF-7:2A cells to estradiol-induced apoptosis that is mediated, in part, by the mitochondrial pathway and also activation of the c-Jun N-terminal kinase (JNK) signaling pathway. We further show that BSO, either alone or in combination with estradiol, causes tumor regression of MCF-7:2A cells *in vivo*.

Materials and methods

Cell lines and reagents

The MCF-7 human breast cancer cell line was obtained from Dr Dean Edwards (University of Texas, San Antonio, TX, USA) and was maintained in phenol red RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 × non-essential amino acids and bovine insulin at 6 ng/mL. The clonal cell line, MCF-7:2A, was derived by growing MCF-7 cells in estrogen-free media for more than 1 year, followed by two rounds of limiting dilution cloning [7]. These cells were grown in phenol red-free RPMI 1640 medium supplemented with 10% 4 × dextran-coated, charcoal-treated FBS (SFS). All reagents for cell culture were obtained from Invitrogen (Life Technologies, Carlsbad, CA, USA). DL-Buthionine sulfoximine (BSO) and 17β-estradiol (E_2) were from Sigma (St Louis, MO, USA), rhodamine 123 (Rh123) was from Invitrogen (Life Technoligies, Carlsbad, CA, USA). LY294002 and SP600125 were from EMD (Gibbstown, NJ, USA)

Western blot analysis

The antibodies used for western blotting included those against stress-activated protein kinase (SAPK)/JNK, phospho-SAPK/JNK (Thr183/Tyr185), caspase-7, caspase-9, phospho-Bcl-2 (Ser70), and poly(ADP-ribose) polymerase (PARP) (Cell Signaling Technology, Danvers, MA, USA), cytochrome *c* and β-actin (Sigma, St Louis, MO, USA), cytochrome oxidase subunit IV (Cox IV; Invitrogen, Carlsbad, CA, USA), Bax, Bcl-2, and Bcl-xL (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Western blotting analysis was performed as previously described [8].

Cell proliferation assays

Proliferation assay was performed as previously described [8]. Briefly, MCF-7 and MCF-7:2A cells were seeded in estrogen-free RPMI media containing 10% SFS at a density of 2×10^4 cells per well in 24-well plates. After 24 h, cells were treated with the respective drugs for 2, 5, and 7 days with retreatment on alternate days. The DNA content of the cells was determined as previously described [25] using a Fluorescent DNA Quantitation kit (Bio-Rad, Hercules, CA, USA). For each analysis, six replicate wells were used, and at least three independent experiments were performed.

Cell proliferation was also determined by cell counting using a hemocytometer. MCF-7 and MCF-7:2A cells were seeded at a density of 0.5×10^6 cells in 100 mm dishes and after 24 h cells were treated with 1 nM E_2, 100 μM BSO, or 1 nM E_2 plus 100 μM BSO for 7 days with re-treatment on alternate days. For each analysis, three replicate dishes were used, and at least three independent experiments were performed.

Detection of apoptosis by annexin V staining

The annexin V-fluorescein isothiocyanate (FITC) labeled Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA) was used to detect and quantify apoptosis by flow cytometry, according to the manufacturer's instructions.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining for apoptosis

Apoptosis was also determined by the TUNEL assay using an *in situ* cell death detection kit conjugated with horse-radish peroxidase (POD) (Roche Applied Science, Indianapolis, IN, USA), according to the manufacturer's instructions. Briefly, fixed cells were washed, permeabilized, and then incubated with 50 μL of terminal deoxynucleotidyl transferase end-labeling cocktail for 60 min at 37°C in a humidified atmosphere in the dark. For signal conversion, slides were incubated with 50 μL of converter-POD (anti-fluorescein antibody conjugated with horseradish peroxidase) for 30 min at 37°C, rinsed with PBS, and then incubated with 50 μL of 3,3'-diaminobenzidine (DAB) substrate solution for 10 min at 25°C. The slides were then rinsed with phosphate-buffered saline (PBS), mounted under glass coverslips, and analyzed under a light microscope using an inverted Nikon TE300 (Nikon, Melville, NY, USA).

GSH assay

Total cellular GSH was measured using the Total Glutathione Colorimetric microplate assay Kit (Oxford Biomedical Research), according to the manufacturer's protocol. Cells were plated at 0.5×10^6/well of a six-well plate and allowed to recover overnight. After appropriate treatments, cells were washed in PBS and then lysed in 100 to 150 μl of buffer (100 mM $NaPO_4$, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5) containing 0.1% Triton X-100 and frozen at -80°C until analysis. To measure total glutathione, proteins were precipitated with sulfosalicylic acid at a final concentration of 1%. Samples were then spun for 10 min in a microcentrifuge to pellet proteins, and supernatant was diluted 1:20 in buffer before being measured. For all measurements, 50-μl triplicates of each sample were used for glutathione determination. The GSH level was obtained by comparing with the GSH standards and represented as nmol/mg of protein.

Mitochondrial transmembrane potential ($\Delta\Psi_m$) and cytochrome c release

Changes in the mitochondrial membrane potential ($\Delta\Psi$m) were examined by monitoring the cells after staining with rhodamine 123. Briefly, estradiol plus BSO-treated MCF-7:2A cells were washed twice with PBS and incubated with 1 μg/mL rhodamine 123 at 37°C for 30 min. Cells were then washed twice with PBS, and Rh123 intensity was determined by flow cytometry. Cells with reduced fluorescence were counted as having lost some of their mitochondrial membrane potential.

For cytochrome *c* release assays, cells were lysed in lysis buffer (10 mmol/L *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES; pH 7.5), 10 mmol/L KCl, and 1 mmol/L EDTA) with protease inhibitor cocktail (Sigma), frozen and thawed three times, and centrifuged at 2,000 *g* for 5 min. The supernatants were centrifuged at 10,000 *g* for 15 min at 4°C, and the mitochondrial pellets were dissolved in sodium dodecyl sulfate (SDS) sample buffer, subjected to 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by immunoblotting with monoclonal antibodies against cytochrome *c* and Cox IV.

RNA isolation and quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated using TRI reagent (Invitrogen) according to the manufacturer's protocol. RNA (2 μg) was reverse transcribed to cDNA using the SuperScript II RNase H-reverse transcriptase system (Invitrogen, Carlsbad, CA, USA). Aliquots of the cDNA were combined with the SYBR green kit and primers, and assayed in triplicate by real-time quantitative PCR using a GeneAmp® 5700 Sequence detection system

(Applied Biosystems Inc, Foster City, CA, USA). Quantitation was performed using the comparative threshold cycle (Ct) method with 18S rRNA as the normalization gene, as previously described [8]. GS and GPx2 primers were designed using Primer Express™ software following the manufacturer's guidelines. Primers were synthesized by Applied Biosystems. Quantitative PCR was performed using the following conditions: 40 cycles; denaturation at 95 C for 15 s, annealing at 63 C for 1 min, and polymerization at 72 C for 1 min. Primer sequences were: GS forward: CACCAGCT GGGGAAG-CATCT; reverse: GGTGAGGGGAAGAGCGT GAA, GPx2 forward: TTG ATT AAG GCT TTC TTT GGT AGG; reverse: TTT CAA TAA ATC AGG TCC CAG G.

Small interfering RNA (siRNA) transfection

Bcl-2-specific siRNA was chemically synthesized by Dharmacon Inc (Chicago, IL, USA). A non-targeting siRNA duplex was used as negative control. For transfection, MCF-7:2A cells were seeded in complete medium without antibiotics the day before the experiment in 12-well plates at a density of 70,000 cells per well. After 24 h, cells were transfected with 100 nM of Bcl-2 siRNA or control siRNA, using DharmaFect 1 transfection reagent (Dharmacon Inc, Chicago, IL, USA), according to the manufacturer's protocol. The cells were harvested 48 h post transfection and analyzed by western blot. Transfected cells were also treated with estradiol for an additional 72 h and apoptotic cells were measured using annexin V staining.

Inhibition of MCF-7:2A cell tumorigenesis by BSO in nude mice

Female CrTac:NCr-Foxn1nu athymic mice (4 to 5 weeks old) were purchased from Taconic (Germantown, NY, USA). Animal experiments were conducted at the Fox Chase Cancer Center (Philadelphia, PA, USA). The research protocol was approved, and mice were maintained in accordance with institutional guidelines of the Fox Chase Cancer Center Animal Care and Use Committee. Mice were acclimatized to the animal facility for 1 week before they received injections of MCF-7:2A human breast cancer cells: 2×10^7 cells were resuspended in 100 μL PBS (Collaborative Biomedical Products, Bedford, MA, USA) and were bilaterally injected into the mammary fat pads of 20 ovariectomized mice. Tumors were allowed to develop for 20 days until they reached a mean cross-sectional area of 0.32 cm^2, when treatment was initiated with placebo (saline), E_2 (0.3 cm capsule), BSO (4 mmol/kg weight), or BSO (4 mmol/kg weight) plus E_2 (0.3 cm capsule) for an additional 7 days. For the estradiol treatment, 0.3 cm silastic estradiol capsules (Baxter HealthCare, Mundeleine, IL, USA) were implanted subcutaneously in the mice. These capsules produced a mean serum estradiol level of 83.8 pg/mL [26], to achieve postmenopausal serum levels of estradiol. BSO was dissolved in saline and was administered intraperitoneally daily for 7 days. The cross-sectional tumor area was calculated by multiplying the length (l) by the width (w) by π and dividing the product by 4 ($lw\pi/4$). Animals were given food and water *ad libitum*. Mice from each group (n = 5) were killed at the conclusion of the experiment and immunohistochemical analysis was performed.

Tissue preparation and immunohistochemistry

Tumors from mice treated with placebo, E_2, BSO, or BSO plus E_2 were excised and fixed in 10% formalin, embedded in paraffin wax blocks and sectioned. Subsequently, sections of the tumor blocks were stained with hematoxylin and eosin (H&E), Ki67, or PARP antibody (1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) by the pathology core facility at Fox Chase Cancer Center.

Statistical analysis

Statistical analysis was performed using the Student t test, and a p value of < 0.05 was considered significant. Data are expressed as the mean ± standard error of the mean (SEM). The mean value was obtained from at least three independent experiments.

Results

Estrogen deprivation increases glutathione levels in MCF-7:2A breast cancer cells

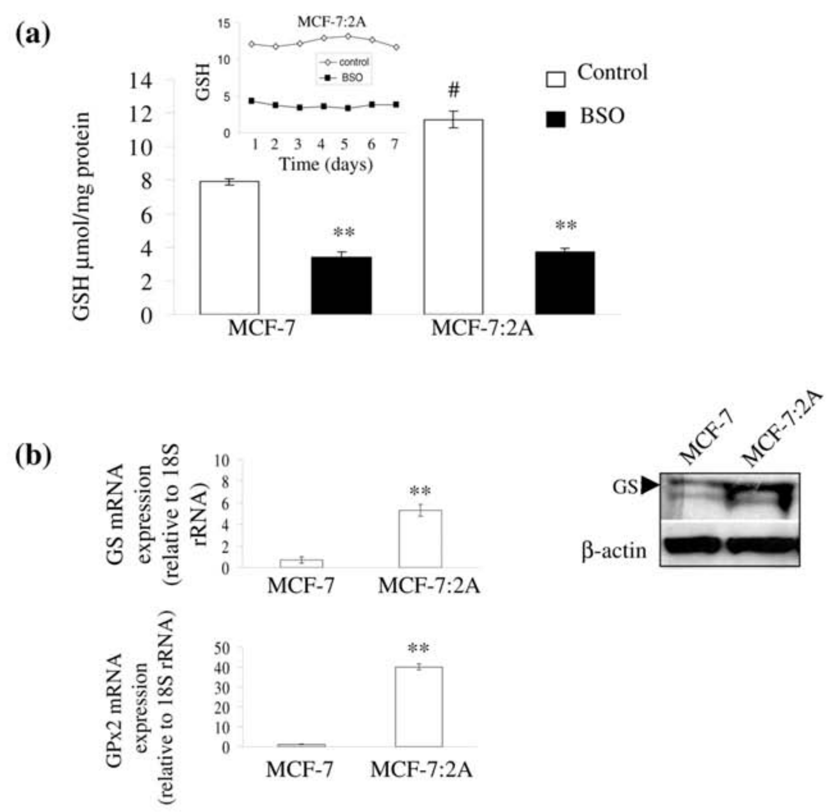
Elevated glutathione levels and the activity of its related enzymes have been characterized as one of the factors which could render breast cancer cells resistant to apoptosis. We have previously shown that MCF-7:2A breast cancer cells are resistant to estrogen-induced apoptosis [7], therefore we measured glutathione levels in these cells along with parental MCF-7 cells. Figure 1a showed that glutathione levels were significantly higher in MCF-7:2A cells (11.9 μM/mg protein) compared to MCF-7 cells (7.8 μM/mg protein) and treatment with BSO (100 μM), an inhibitor of glutathione synthesis, for 24 h depleted glutathione content by approximately 55% and 68% in MCF-7 and MCF-7:2A cells, respectively. It is worth noting that glutathione levels were consistently elevated in MCF-7:2A cells up to 7 days and the inhibitory effect of BSO persisted throughout that incubation period (Figure 1a, insert).

We next examined whether the expression of glutathione-related enzymes was altered in these cells. Using quantitative real-time PCR, we found a 6-fold increase in glutathione synthetase (GS) expression and a 40-fold increase in glutathione peroxidase 2 (GPx2) expressions in MCF-7:2A cells compared to parental MCF-7 cells (Figure 1b). Western blot analysis also showed a marked increase in GS protein level in MCF-7:2A cells compared to parental MCF-7 cells (Figure 1b, right panel).

BSO enhances the apoptotic effect of E_2 in MCF-7:2A cells

We next examined whether depletion of glutathione levels by BSO sensitizes MCF-7:2A cells to estrogen-induced apoptosis. For proliferation assays, MCF-7 and MCF-7:2A cells were

Figure 1



Intracellular glutathione (GSH) levels in wild-type MCF-7 cells and antihormone-resistant MCF-7:2A breast cancer cells. (a) MCF-7 and MCF-7:2A cells were seeded at 2 × 10^6 cells per 100 mm culture plates in phenol red RPMI media containing 10% fetal bovine serum (FBS) and phenol red-free RPMI media containing 10% 4× dextran coated charcoal-treated FBS (SFS), respectively, and after 24 h were treated with nothing (control) (white columns) or 100 µM buthionine sulfoximine (BSO) (black columns) for 24 h. Total cellular glutathione was measured using a Glutathione Colorimetric microplate assay kit, as described in Materials and methods. Columns, mean from three separate experiments; bars, ± standard error of the mean (SEM). **, $p < 0.001$ compared with control cells; #, $p < 0.05$ compared with MCF-7 control cells. Insert graph shows glutathione levels in MCF-7:2A cells over a 7-day period. (b) Quantitative real-time polymerase chain reaction (PCR) of glutathione sythetase (GS) (top left) and glutathione peroxidase 2 (GPx2) (bottom left) mRNA expression in MCF-7 and MCF-7:2A cells. **, $p < 0.001$ compared with MCF-7 control cells. Western blot analysis of GS protein expression in MCF-7:2A cells is also shown (top right).

seeded in estrogen-free media, and after 24 h, were treated with 100 µM BSO, 1 nM E_2, or 100 µM BSO plus 1 nM estradiol for 2, 5, and 7 days. Figure 2a shows that the growth of parental MCF-7 cells was stimulated sevenfold over the control cells by 1 nM estradiol during the course of the 7-day assay and that treatment with BSO, either alone or in combination with estradiol, did not significantly alter the growth of these cells. In contrast, MCF-7:2A cells treated with the combination of 100 µM BSO and 1 nM estradiol showed a significant time-dependent decrease in cell growth relative to cells treated with either estradiol or BSO alone. The growth inhibitory effect of BSO and estradiol was observed as early as 48 h after treatment and persisted over the time course of the experiment with maximum cell death at the 7-day time point. The combination of estradiol plus BSO also significantly reduced the proliferation of MCF-7:2A cells (Fig. 2c, bottom) but it did not affect the growth of wild type MCF-7 cells (Figure 2c, top). Furthermore, we found that treatment with the antiestrogen 4-hydroxytamoxifen (4-OHT) almost completely reversed the growth inhibitory effect of estradiol and BSO in MCF-7:2A cells (see Additional data file 1) which suggests the involvement of the ER in this process.

Based on the above finding, we next determined whether MCF-7:2A cells underwent apoptotic cell death upon BSO and estradiol treatment. We performed a TUNEL assay, which detects the fragmentation of DNA, which is characteristic of cells undergoing apoptotic cell death. As shown in Figure 3a, the percentage of TUNEL-positive cells significantly increased with the combination of BSO and estradiol but not with estradiol or BSO alone. After treatment with BSO and estradiol (96 h), as many as 53% of cells displayed TUNEL-positive staining, whereas, only 1% of the control cells and 5% of the estradiol-treated cells were TUNEL-positive. BSO-treated cells

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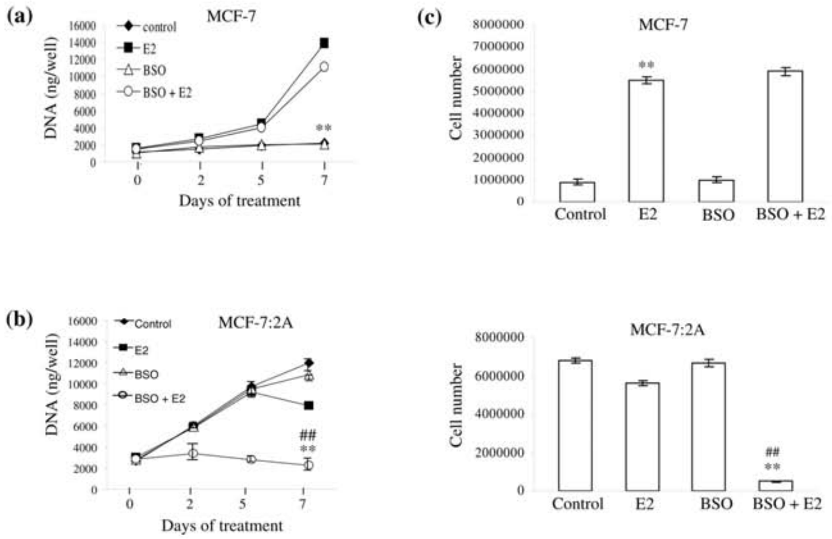
Figure 2



Effect of buthionine sulfoximine (BSO) plus estradiol on the growth of wild-type MCF-7 cells and antihormone-resistant MCF-7:2A cells. (a) MCF-7 cells were grown in estrogen-free media for 3 days prior to the start of the growth assay. On the day of the experiment, 30,000 cells were seeded in 24-well plates and after 24 h were treated with $< 0.1\%$ ethanol vehicle (control), 1 nM 17β-estradiol (E_2), 100 μM BSO, or 100 μM BSO plus 1 nM E_2 for 7 days. At the indicated time points, cells were harvested and total DNA (ng/well) was quantitated as described in Materials and methods. The data represent the mean of three independent experiments; bars, ± standard error of the mean (SEM). **, $p < 0.001$ compared with control cells. (b) MCF-7:2A cells were seeded at the same density as MCF-7 cells and were treated similarly. The data represent the mean of three independent experiments; bars, ± SEM. **, $p < 0.001$ compared with control cells; ##, $p < 0.001$ compared with estradiol-treated cells. (c) The effect of BSO plus estradiol on cell proliferation was also determined by cell counting using a hemocytometer. For experiment, 0.5×10^6 MCF-7 (top) and MCF-7:2A (bottom) cells were seeded in 15-cm dishes and after 24 h were treated with 1 nM estradiol, 100 μM BSO, or E_2 plus BSO combination for 7 days. Data shown represents the mean of three independent experiments; bars, ± SEM. **, $p < 0.001$ compared with control cells; ##, $p < 0.001$ compared with estradiol-treated cells.

looked similar to control cells. As expected, parental MCF-7 cells showed very little TUNEL-positive staining in the presence of estradiol alone or BSO plus estradiol combined (Figure 2b, top panel), thus indicating a lack of apoptosis in these cells.

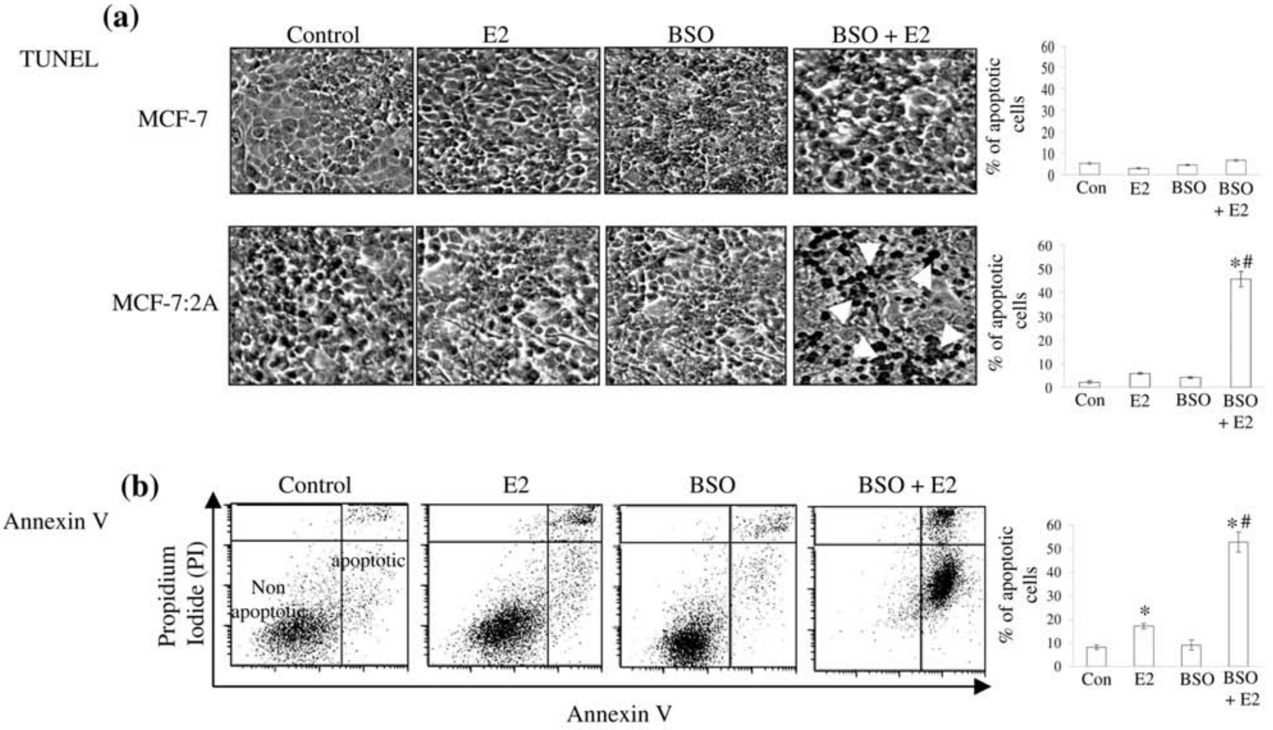
To further substantiate the apoptotic effect of BSO and estradiol in MCF-7:2A cells, annexin V-PI immunostaining was performed by flow cytometry. Figure 3b shows that in the BSO plus estradiol-treated group, approximately 55.6% of cells stained positive for annexin V whereas in the control group and estradiol-treated group, approximately 7.4% and approximately 15.6%, respectively, of cells stained positive for annexin V. For the BSO-treated group, only 8.7% of cells stained positive for annexin.

Role of the mitochondrial pathway in BSO plus estradiol-induced apoptosis in MCF-7:2A cells

To examine the role of the mitochondrial pathway in BSO plus estradiol-induced apoptosis, western blot analyses was used to measure Bax, Bcl-2, phosphorylated Bcl-2, and Bcl-xL protein levels in MCF-7:2A cells following treatment with 1 nM estradiol alone, 100 μM BSO, or BSO plus estradiol for 48 h. We found that Bcl-2, phospho-Bcl-2, and Bcl-xL protein levels were almost completely reduced in MCF-7:2A cells treated with BSO plus estradiol compared to control, BSO, or estradiol alone. In addition, a marked increase in Bax expression was also observed in MCF-7:2A cells following BSO plus estradiol combined treatment (Figure 4a). In contrast, similar experiments performed with parental MCF-7 cells showed that BSO plus estradiol slightly increased Bcl-2 and phospho-Bcl-2 protein levels in these cells with a more dramatic effect observed with estradiol alone (Figure 4a). It is worth noting that in MCF-7:2A cells endogenous levels of Bcl-2 and phosphorylated Bcl-2 were markedly elevated compared to parental MCF-7 cells. This finding is consistent with previous reports which show that overexpression of Bcl-2 increases glutathione levels and inhibits mitochondrial dysfunction and cell death elicited by glutathione-depleting reagents [27].

Although estradiol, as an individual treatment, did not significantly induce apoptosis in MCF-7:2A cells, it did decrease Bcl-2 protein level in these cells. We therefore tested whether siRNA knockdown of Bcl-2 expression would sensitize MCF-7:2A cells to estradiol-induced apoptosis. Expression of Bcl-2 following knockdown was analyzed by western blotting. As expected, Bcl-2 protein levels were significantly reduced following transfection of MCF-7:2A cells with Bcl-2 siRNA com-

Figure 3



Buthionine sulfoximine (BSO) plus estradiol induce apoptosis in MCF-7:2A cells. (a) Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining for apoptosis in MCF-7:2A cells following BSO plus 17β-estradiol (E_2) treatment for 96 h were performed as described in Materials and methods. Slides were photographed through a brightfield microscope under 100 × magnification. TUNEL-positive cells were stained black (white arrows). Columns (right), mean percentage of apoptotic cells (annexin V-positive cells) from three independent experiments performed in triplicate; bars, ± standard error of the mean (SEM). *, $p < 0.001$ compared with control cells; $^\#$, $p < 0.001$ compared with estradiol-treated cells. (b) Annexin V staining for apoptosis. Cells were seeded in 100 mm plates at a density of 1×10^6 per plate and after 24 h were treated with ethanol vehicle (control), 1 nM E_2, or BSO plus E_2 for 72 h and then stained with fluorescein isothiocyanate (FITC)-annexin V and propidium iodide (PI) and analyzed by flow cytometry. PI was used as a cell viability marker. Representative cytograms are shown for each group. Quantitation of apoptosis (percentage of control) in the different treatment groups is shown on the right. bars, ± SEM. *, $p < 0.05$ compared with control cells; $^\#$, $p < 0.01$ compared with estradiol-treated cells.

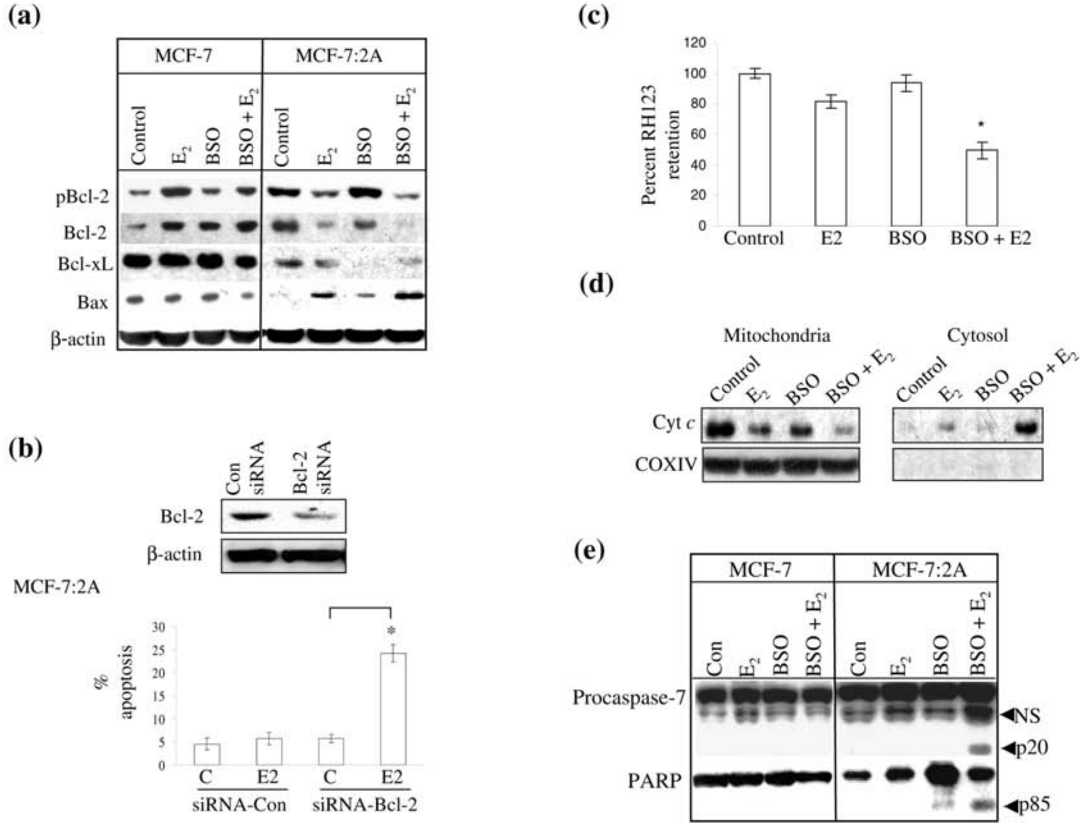
pared to control siRNA (Figure 4b, top panel). Using annexin V staining, we found that apoptosis was increased by 20% in Bcl-2 siRNA transfected cells compared with cells transfected with the control siRNA (Figure 4b, bottom panel), thus suggesting that suppression of antiapoptotic factors such as Bcl-2 has the ability to partially sensitize hormone-independent MCF-7:2A cells to apoptosis.

We next examined mitochondrial membrane integrity using the Rh123 retention assay. Cells were treated with nothing (control), estradiol, BSO, or BSO plus estradiol for 48 h. Figure 4c shows that BSO plus estradiol treatment reduced Rh123 fluorescence in MCF-7:2A cells by approximately 50% compared to control, whereas, estradiol or BSO, as individual treatments, did not significantly alter Rh123 retention levels in these cells. BSO plus estradiol also enhanced cytochrome *c* release in MCF-7:2A cells. Figure 4d shows that in the control cells, cytochrome *c* was detected primarily in the mitochondria and was undetectable in the cytosol; however, in the presence of BSO plus estradiol (48 h), all of cytochrome *c* was observed in the cytosol. BSO or estradiol, as individual treatments, did not significantly alter mitochondrial release of cytochrome *c*. The translocation of cytochrome *c* from the mitochondria to the cytosol following BSO plus estradiol treatment coincided with cleavage of caspase 7 and PARP (Figure 4e), which is a molecular signature of apoptosis. Cleavage of PARP and caspase 7 was blocked by the pan-caspase inhibitor z-VAD (data not shown).

The apoptotic effect of BSO and estradiol in MCF-7:2A cells is regulated, in part, by JNK signaling

Emerging evidence supports a role for JNK in stress-induced mitochondrial apoptotic pathways in a variety of cell systems [28]. Therefore, we examined the possible involvement of c-Jun/JNK pathway in BSO plus estradiol-induced apoptosis in MCF-7:2A cells. JNK activation was determined by western

Figure 4



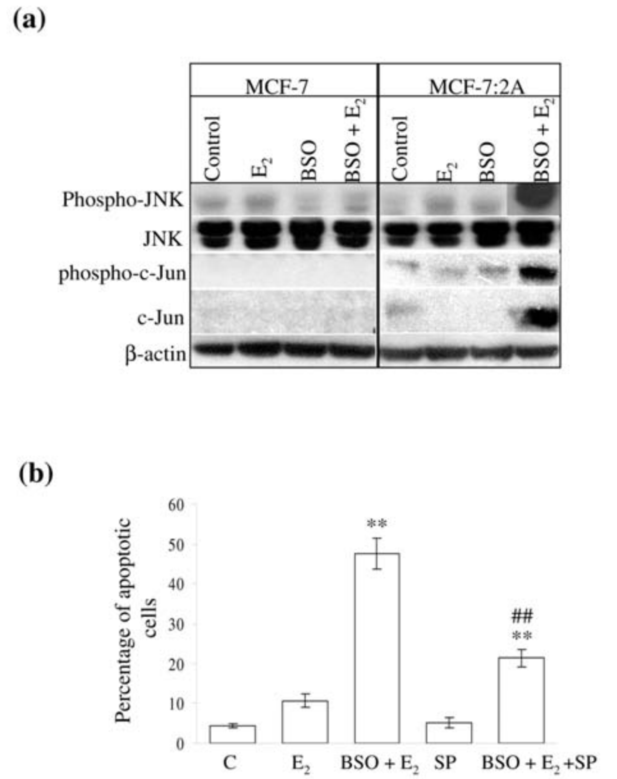
Effect of buthionine sulfoximine (BSO) and 17β-estradiol (E_2) on Bcl-2 family protein expression and mitochondrial function in MCF-7 and MCF-7:2A cells. (a) Western blot analysis for pBcl-2, Bcl-2, Bcl-x_L, and Bax protein expression in parental MCF-7 cells and MCF-7:2A cells following 48 h of treatment with ethanol vehicle (Control), 1 nM E_2, 100 μM BSO, or E_2 + BSO. Equal loading was confirmed by reprobing with an antibody against β-actin. (b) Small interfering RNA (siRNA) knockdown of Bcl-2 partially sensitizes MCF-7:2A cells to E_2-induced apoptosis. Cells were transfected with 100 nM siRNA-Bcl-2 or siRNA-Con (control) and expression levels of Bcl-2 was determined by immunoblot analysis (top). Annexin V staining (bottom) showing the effects of siRNA-con and siRNA-Bcl-2 on apoptosis induced by estradiol treatment in MCF-7:2A cells. *, $p < 0.001$. (c) Loss of mitochondrial potential in MCF-7:2A cells was determined by rhodamine 123 (Rh123) retention assay. The percentage of cells retaining Rh123 in each treatment group was compared with untreated control. (d) Cytochrome *c* release from the mitochondria to the cytosol after treatment with E_2 alone or BSO and E_2 for 48 h was determined as described in Materials and methods. Anti-Cox IV antibody was used as a control to demonstrate that mitochondrial protein fractionation was successfully achieved. (e) Cleavage of caspase 7 and poly(ADP-ribose) polymerase (PARP) (72 h) was assessed by western blot using specific antibodies. The upper band of caspase 7 represents the full-length protein and the lower band (p20, arrow) represents the cleaved activated product; NS, nonspecific. Full length PARP is approximately 116 kDa; cleaved (active) PARP is 85 kDa (arrow). The results are representative of three independent experiments.

blot analysis after 48-h treatment of cells with BSO plus estradiol. A profound induction of the p54 and p46 isoforms of phosphorylated JNK as well as a significant increase in phospho-c-Jun and c-Jun were observed in MCF-7:2A cells treated with BSO plus estradiol compared to BSO alone or control (Figure 5a). Interestingly, treatment with estradiol alone also significantly increased phosphorylated JNK in MCF-7:2A cells. We also found that pretreatment of MCF-7:2A cells with the JNK inhibitor, SP600125 (20 μM) markedly reduced the apoptotic effect of BSO plus estradiol in these cells (Figure 5b). Overall, these results suggest a possible involvement of the c-Jun/JNK signaling pathway in BSO plus estradiol-induced apoptosis in MCF-7:2A cells.

BSO inhibits the growth of MCF-7:2A cells *in vivo*

To determine whether the effect of BSO plus estradiol was relevant *in vivo*, we used a xenograft model in which MCF-7:2A cells were injected into CrTac:NCr-Foxn1nu athymic mice (n = 20). At 20 days post injection, tumors grew to a mean cross-sectional area of 0.30 cm^2 and mice were randomized to four groups; placebo (saline), estradiol, BSO, or the combination of BSO plus estradiol, as described in materials and methods. After 7 days of treatment, tumor growth was reduced by 25% in mice treated with estradiol alone whereas in the BSO and BSO plus estradiol group tumor growth was reduced by 40% and 60%, respectively, compared to the placebo group which showed a 7% increase in growth (Figure 6a). Interestingly, we

Figure 5



Activation of c-Jun N-terminal kinase (JNK) signaling pathway in MCF-7:2A cells in response to buthionine sulfoximine (BSO) and 17β-estradiol (E_2) treatment. (a) MCF-7 and MCF-7:2A cells were treated with ethanol vehicle (control), 1 nM E_2 or 100 μM BSO plus E_2 for 48 h and protein levels of phosphorylated JNK, JNK, phosphorylated c-Jun, and c-Jun were analyzed by western blotting. β-Actin was used as a control. (b) Inhibition of JNK activation by SP600125 (SP) partially reverses the apoptotic effect of BSO and estradiol in MCF-7:2A cells. Cells were pretreated with 20 μM SP600125 or vehicle for 24 h, then further incubated for 48 h with 1 nM E_2, E_2 + 100 μM BSO, 20 μM SP, or E_2 + BSO + SP and apoptosis was determined by annexin V-propidium iodide (PI) staining as described in Materials and methods. Columns, mean percentage of apoptotic cells from three independent experiments performed in triplicate; bars, ± standard error of the mean (SEM). **, $p < 0.001$ compared with control (C) cells; ##, $p < 0.01$ compared with E_2 plus BSO-treated cells.

found that BSO *in vitro* had a relatively small effect on growth, however, *in vivo* its effect was very pronounced, thus suggesting the possibility of altered glutathione metabolism *in vivo*. We performed histology on tumors taken from placebo, estradiol, BSO, or BSO plus estradiol groups at day 27. H&E staining of the BSO plus estradiol-treated tumors revealed less tumor cells and more intercellular matrix, significantly less mitoses, chromatin clumping and dark staining which are associated with apoptosis, and enhanced abnormalities in shape and size, compared to tumors from placebo or BSO or estradiol-treated groups (Figure 6b). We also characterized the proliferative status of these cells by staining tumors for the expression of Ki67, a marker of cell proliferation. We observed a 32% decrease ($p < 0.001$) in the number of Ki67 stained tumors from the BSO plus estradiol-treated group and a 21% decrease in the BSO-treated group compared to the placebo group whereas estradiol treatment caused an 8% increase in Ki67 staining (Figure 6c). Immunohistochemistry of paraffin-embedded tumor sections of mice treated with the combination of BSO and estradiol showed increased immunostaining for proteolytically cleaved PARP (marker for apoptosis) compared to control, estradiol, or BSO-treated groups (Figure 6d). Overall, these data show that BSO either alone or in combination with estradiol, reduces tumor growth by inhibiting proliferation and increasing apoptosis.

Discussion

In the current study, we investigated whether suppression of the antioxidant glutathione by BSO has the ability to sensitize antihormone resistant MCF-7:2A breast cancer cells to estradiol-induced apoptosis. Our results showed that glutathione levels and the enzymes involved in its synthesis, glutathione synthetase and glutathione peroxidase, were significantly elevated in MCF-7:2A cells compared to parental MCF-7 cells and that suppression of glutathione by BSO sensitized these cells to estrogen-induced apoptosis *in vitro* and *in vivo*. The BSO-mediated estradiol-induced apoptosis was associated with a marked decrease in the expression of antiapoptotic Bcl-2 and Bcl-xL proteins and a significant increase in proapoptotic Bax protein. It is worth noting that high-dose estrogen was generally considered the endocrine therapy of choice for postmenopausal women with breast cancer prior to the introduction of tamoxifen, however, due to undesirable side effects, the use of high-dose estrogen was largely abandoned [29]. Here, we show that the killing effect of estradiol in antihormone resistant cells can be achieved at physiological concentrations when it is combined with non-toxic concentrations of BSO. Our present findings are consistent with previous studies which have shown that the cytotoxicity of a number of chemotherapeutic drugs, including melphalan [30], doxorubicin [31], and bleomycin [32], are significantly enhanced when glutathione is depleted by BSO.

An important target of BSO plus estradiol-induced apoptosis appears to be Bcl-2, whose protein expression was dramatically decreased in MCF-7:2A cells following glutathione depletion. Previous studies have shown that Bcl-2 functions as an antioxidant to block apoptosis and that Bcl-2 protein levels and glutathione intracellular concentration is coordinately regulated with a decrease in either favoring cell death [23,33]. It is believed that one mechanism by which Bcl-2 may function as an antioxidant is through upregulation of glutathione, leading to rapid detoxification of reactive oxygen species and inhibition of free radical-mediated mitochondrial damage. Bcl-2 also has the ability to shift the entire cellular redox potential to a more reduced state, which is independent of its effect on glutathione levels [33]. It is worth noting that glutathione levels

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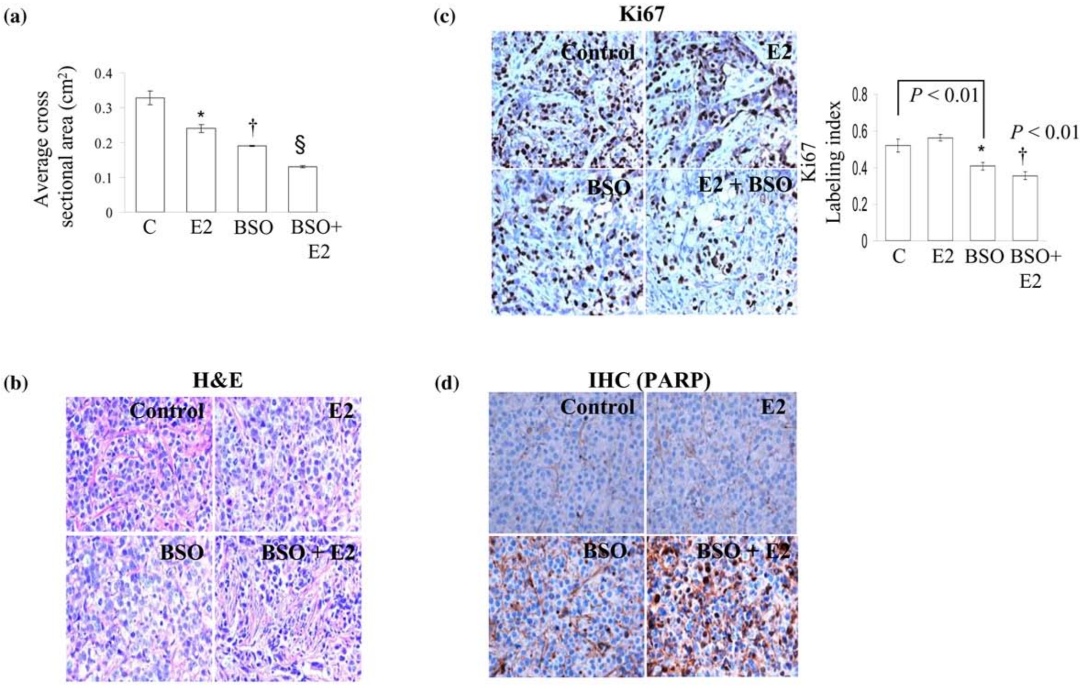
Figure 6



Buthionine sulfoximine (BSO) inhibits the growth of MCF-7:2A tumors in vivo. Athymic nude mice (4 to 5 weeks old, n = 20) were injected with MCF-7:2A breast cancer cells and after 20 days when tumors had reached a mean cross-sectional area of 0.3 cm^2, animals were randomized into 4 groups and were treated with placebo (saline), 17β-estradiol (E_2), BSO, or BSO plus E_2 for 7 days as described in Materials and methods. BSO (4 mmol/kg weight) was diluted in saline and was injected intraperitoneally daily. (a) Tumor size was measured everyday and cross-sectional area was calculated by multiplying the length (l) by the width (w) by π and dividing the product by 4 ($lw\pi/4$). Data is shown as mean ± standard error of the mean (SEM). *, $p < 0.05$, control group compared with the E_2 group; †, $p < 0.002$ control group compared with BSO group; § $p < 0.001$ control group compared with BSO + E2 group. (b) Microscopy of hematoxylin and eosin (H&E)-stained histological sections of MCF-7:2A tumors treated with placebo, E_2, BSO, or BSO plus E_2. (c) Immunohistochemical analysis of the proliferation marker Ki-67 in MCF-7:2A tumors treated with placebo, E_2, BSO, or BSO plus E_2. (d) Paraffin-embedded tumor sections of mice treated with E_2, BSO, or BSO plus E_2 were immunostained for proteolytically cleaved poly(ADP-ribose) polymerase (PARP), which exists only when cells undergo apoptosis. Three to four tumors per treatment group were analyzed.

and Bcl-2 protein expression were significantly elevated in MCF-7:2A cells compared to parental MCF-7 cells. In phase I trials [34,35], the concentration of BSO in blood has been shown to reach 0.5 to 1 mM, whereas, in mice [36,37] the concentration has been estimated to be 5 to 6 mM following an *in vivo* treatment of 4 mmol/kg. In our study, we showed that 100 μM BSO decreased glutathione concentrations by approximately 60% after 24 h and that BSO enhanced the apoptotic effect of estradiol in MCF-7:2A breast cancer cells as early as 48 h after treatment. Interestingly, treatment with BSO alone did not cause apoptosis in MCF-7:2A cells, indicating that glutathione depletion alone may not trigger apoptosis in these cells. This finding is consistent with previous studies by Mirkovic *et al.* [38] which showed that inhibition of glutathione by BSO did not increase susceptibility of mouse lymphoma cells to radiation-induced apoptosis even under conditions where glutathione levels were lowered by 50%. Other groups have made similar observations using BSO [39]. One possible explanation for this apparent contradiction might be the fact that BSO does not lower glutathione levels in mitochondria as effectively as it does in the cytoplasm [40]. Mitochondrial glutathione concentrations are regulated and have been implicated in apoptotic cell death [41], hence, it would be of interest to evaluate relative glutathione concentrations in the mitochondrial matrix of MCF-7:2A cells following treatment with BSO either alone or in combination with estradiol. Another possibility could be that cellular thiols other than glutathione may play important roles in regulating apoptosis [39]. The flavoprotein thioredoxin has been shown to be upregulated in several human tumors and is implicated in both cancer cell growth and apoptotic resistance [42]. However, it is not known whether Bcl-2 or other apoptotic regulators can influence the levels of thioredoxin or whether such modulation may contribute to resistance in human tumor cells.

Apart from Bcl-2, we also found that proapoptotic Bax protein was markedly increased in MCF-7:2A cells by the combination of BSO plus estradiol and this induction coincided with a loss of mitochondrial membrane integrity and cytochrome *c* release. Bax is normally found as a monomer in the cytosol of non-apoptotic cells and it oligomerizes and translocates to the outer mitochondrial membrane in response to apoptotic stimuli and induces mitochondrial membrane permeabilization and cytochrome *c* release [19]. In MCF-7:2A cells, Bax protein was induced as early as 24 h after BSO plus estradiol treatment (Figure 4) and suppression of Bax expression using siRNA was able to partially reverse the apoptotic effect of the combination treatment (data not shown). The induction of Bax coincided with cytochrome *c* release from the mitochondria into the cytosol, which was followed by activation of caspase 7, and PARP cleavage. It is worth noting that pretreatment of cells with the universal caspase inhibitor z-VAD almost completely blocked the apoptotic effect of BSO plus estradiol. It is also worth noting that antiapoptotic Bcl-2 and Bcl-xL proteins were also markedly decreased in MCF-7:2A cells following the combination treatment of BSO plus estradiol (Figure 4) and overexpression of Bcl-xL partially blocked the apoptotic effect of BSO plus estradiol (data not shown). This finding is important because there is evidence that suggests that the ratio rather than the amount of antiapoptotic vs proapoptotic proteins determines whether apoptosis will proceed [43]. Thus, it is reasonable to suggest that the apoptotic effect of BSO plus estradiol is mediated, in part, by the mitochondrial pathway through their ability to alter the ratio between proapoptotic and antiapoptotic proteins in target cells.

In addition to the mitochondrial pathway, BSO plus estradiol appears to induce apoptosis, in part, through activation of the JNK signaling pathway. JNKs are a group of mitogen-activated protein kinases (MAPKs) that bind the N-terminal activation domain of the transcription factor c-Jun and phosphorylate c-Jun on amino acid residues Ser63 and Ser73 [44]. JNKs are stimulated by multiple factors including cytokines, DNA-damaging agents, and environmental stresses and are important in controlling programmed cell death or apoptosis. The inhibition of JNKs has been shown to enhance chemotherapy-induced inhibition of tumor cell growth, suggesting that JNKs may provide a molecular target for the treatment of cancer [44]. We found that JNK activation (as measured by the increased levels of phospho-JNK1/2 and the JNK substrate phospho-c-Jun) correlated well with BSO plus estradiol-induced apoptosis in MCF-7:2A cells and pharmacologic disruption of this pathway using the JNK inhibitor SP600125 significantly attenuated this effect. Previously, Chen and coworkers [45] reported that BSO enhanced the apoptotic effect of arsenic (As_2O_3) in leukemia and lymphoma cells through activation of JNK and upregulation of death receptor (DR)5 and that inhibition of JNK by SP600125 decreased DR5 upregulation and apoptotic induction in U937 leukemia cells treated with arsenic plus BSO. While the exact mechanism by which JNK promotes apoptosis is not currently known, the phosphorylation of transcription factors such as c-Jun and p53, as well as pro- and antiapoptotic Bcl-2 family members [46] has been suggested to be of importance. It is worth noting that treatment with BSO plus estradiol markedly increased phosphorylated c-Jun in MCF-7:2A cells and decreased phosphorylated Bcl-2 in these cells. These findings thus suggest that BSO plus estradiol might mediate their apoptotic effect, in part, through activation of JNK.

Conclusion

We have demonstrated that glutathione depletion by BSO sensitizes hormone-resistant MCF-7:2A human breast cancer cells to estradiol-induced apoptosis *in vitro* and *in vivo*. This finding has important clinical implications; particularly for the use of estrogen deprivation as long-term therapy, and it suggest that, if and when resistance develops, a strategy of treatment with estrogen combined with BSO may be effective in sensitizing resistant cells to apoptosis. It is worth noting that recently, Lonning and coworkers [9] reported a 33% complete response (that is, stable disease) with high dose diethylstilbestrol (DES) in postmenopausal patients with advanced breast cancer who were heavily pretreated with endocrine agents. However, 67% of the patients showed partial or no response [9] so the key to future clinical progress in the treatment of antihormone resistant breast cancer is to improve current treatment strategies. We are currently evaluating the optimal dose of daily estradiol therapy to reverse antihormonal resistance [4] but the goal is to enhance the estradiol-induced apoptotic response. The present findings suggest that BSO is indeed capable of enhancing the apoptotic effect of estradiol in antihormone resistant breast cancer cells. It is worth noting that a phase I study of BSO administered with the anticancer drug melphalan showed that continuous-infusion of BSO was relatively nontoxic and resulted in depletion of tumor glutathione [35,47]. Thus it is possible that future clinical studies of BSO infusions combined with low dose estrogen hold the promise of improving disease control for patients with antihormone resistant ER-positive metastatic breast cancer.

Competing interests

The authors declare that they have no competing interests. The views and opinions of the author(s) do not reflect those of the US Army or the Department of Defense.

Authors' contributions

JSLW designed and coordinated the studies, analyzed the data and interpreted the results, generated the figures, and wrote and revised the manuscript. HK performed the cell proliferation assays and the western blots. CW performed the glutathione assay. RP and JP performed the animal experiments. AJK performed the immunohistochemistry. VCJ is the Principal Investigator (PI) of the laboratory in which all experiments were conducted and is the recipient of the grant that partially funded the project. VCJ was instrumental in revising the manuscript. All authors read, assisted in revision and approved the final manuscript.

Additional files

The following Additional files are available online:

Additional file 1

Powerpoint file showing the growth inhibitory effect of buthionine sulfoximine (BSO) and 17β-estradiol (E_2) in MCF-7:2A cells is reversed by the antiestrogen 4-hydroxytamoxifen (4-OHT). MCF-7:2A cells (30,000/well) were seeded in 24-well plates and after 24 h were treated with $< 0.1\%$ ethanol vehicle (control), 1 nM E_2, 100 μM BSO, 100 μM BSO plus 1 nM E_2, 1 μM 4-OHT, 4-OHT + E_2, 4-OHT + BSO, 4-OHT + E_2 + BSO for 7 days. At the indicated time points, cells were harvested and total DNA (μg/well) was quantitated as described in Materials and methods. The data represent the mean of three independent experiments; bars, ± standard error of the mean (SEM). *, $p < 0.01$ compared with control cells; #, $p < 0.01$ compared to E_2-treated cells.
See http://www.biomedcentral.com/content/supplementary/bcr2208-S1.ppt

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Jordan, V.C.

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Short communication

By looking back we can see the way forward: enhancing the gains achieved with antihormone therapy

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Sir Alexander Haddow discovered the first chemical therapy to treat cancer [1]. Based on Paul Ehrlich's pioneering work that resulted in chemical therapy or chemotherapy to treat bacterial infections [2], Haddow investigated the therapeutic potential of numerous polycyclic hydrocarbons to cause tumour regression in experimental animals. Some compounds were effective, but the fact that they were known carcinogens prohibited further exploration in humans. Nevertheless, the triphenylethylene-based oestrogens [3] have a structural similarity to polycyclic hydrocarbons and they were also observed to cause tumour regression in animals. This was the translational basis of Haddow's landmark clinical experiments to evaluate the efficacy of high-dose oestrogen on the growth of breast and prostate cancer. Responses were noted but Haddow later commented [4] in 1970 during the inaugural David A Karnofsky lecture that, 'The extraordinary extent of tumour regression observed in perhaps 1% of postmenopausal cases has always been regarded as of major theoretical importance and it is a matter of some disappointment that so much of the underlying mechanisms continue to elude us.'

High-dose oestrogen therapy was introduced into clinical care during the 1950s [5] for the treatment of postmenopausal women with metastatic breast cancer. This approach complemented the use of ovarian ablation (using radiation at that time) in premenopausal patients, but the observation that high-dose oestrogen was an effective treatment for one in three elderly postmenopausal breast cancer patients remained a mechanistic paradox until recently [6].

Through serendipity, a young endocrinologist, Leonard Lerner at Merrell Dowe Pharmaceuticals in the USA, recognized that a triphenylethanolic compound being tested as a cardiovascular drug had a structure similar to the triphenylethylenes [7]. He asked to test the compound but found that there was no oestrogenic activity in any species tested, only anti-oestrogen activity. The compound, MER25 or ethamoxytriphetol, was the first nonsteroidal anti-oestrogen [8]. However, it was the fact that nonsteroidal anti-oestrogens were postcoital antifertility agents in rats that drove the structural evolution of triphenylethylene-based oestrogens to become a whole range of novel anti-oestrogenic compounds [9]. Regrettably, the promise of preventing pregnancy was premature because the compounds actually induced ovulation [10]. Also, drug toxicities noted during the 1960s and 1970s retarded any serious consideration of the non-steroidal anti-oestrogens as therapeutic agents for indications such as breast cancer therapy [10]. Only ICI 46,474, the *trans* isomer of a substituted triphenylethylene [11], took a tenuous path to clinical testing in breast cancer [10,12] and was subsequently kept on life support to be reinvented [13] as a potential targeted therapy for the long-term adjuvant treatment and prevention for oestrogen receptor positive breast cancer.

Today, the advance with the clinical implementation of the scientific strategy is profound [14,15], and the practice of oncology has progressed significantly over the past three decades [6]. However, the consequences of long-term antihormonal therapy is drug resistance, and it is the laboratory study of the drug resistance of tamoxifen and subsequently the aromatase inhibitors that has provided the opportunity to solve the paradox of high-dose oestrogen therapy in breast cancer. Solving this mystery has had the potential to show the way forward for future advances in cancer care.

Models to study the development of drug resistance to long-term tamoxifen resistance were first reported 20 years ago [16,17]. Drug resistance to tamoxifen develops within about a year in MCF-7 breast cancer cells. Inoculated cells grow into transplantable tamoxifen-stimulated tumours in ovariectomized athymic mice [16], and drug resistance (subsequently also noted for raloxifene [18,19]) is consistent with clinical

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VEGFR = vascular endothelial growth factor receptor.

experience. However, it should be stressed that tamoxifen-stimulated growth is a unique form of drug resistance. Tumours stop growing when tamoxifen is withdrawn, but oestrogen also stimulates tumours to grow. This is the scientific basis for the use of an aromatase inhibitor or fulvestrant, the pure anti-oestrogen, after the development of tamoxifen resistance [20]. However, the finding that tamoxifen resistance actually evolves into new phases [21] provided an experimental basis for solving the mystery of the mechanism of high-dose oestrogen therapy and an opportunity to enhance the effectiveness of antihormonal therapy in patients rendered refractory to multiple anti-oestrogenic treatments.

Tamoxifen-stimulated MCF-7 breast tumours can only be maintained as a model of human disease by serial transplantation into tamoxifen-treated athymic mice; no appropriate cellular model is available. However, the realization that the model does not replicate adjuvant treatment with tamoxifen (5 or more years) raised the question of what occurs under these clinical circumstances. The discovery that physiological oestrogen causes rapid tumour regression of long-term (5 plus years) tamoxifen-resistant MCF-7 tumours [22] and the subsequent finding that the oestrogen-stimulated regrowth of regressed tumour would again respond to the anti-oestrogen tamoxifen [23] indicated a new strategic approach to cancer care. Simply stated, for the first time there was a novel method for killing antihormone-resistant breast cancer cells and then effectively retreating with tamoxifen to maintain responding patients for longer periods. The development of mechanistic studies and the important observations that the principle of oestrogen-stimulated tumour cell regression and apoptosis also applied to oestrogen-deprived cells (aromatase inhibitor resistant) [24-26] enhanced the overall relevance of the observations and provided opportunities for further mechanism based clinical trials.

The important study conducted by Lønning and coworkers [27] provides the laboratory-to-clinic translation of the fact that high-dose oestrogen treatment can produce a response rate of up to 30% among patients who have been treated with exhaustive antihormone therapy. The question now being addressed in multiple clinical studies is whether low-dose oestrogen therapy will be as effective in treating patients with a sensitized breast tumour.

With the evolution of thinking about oestrogen action following Haddow's success with the first chemical therapy [1], it is reasonable to examine how we can improve the efficacy of long-term antihormonal therapy and the putative 30% response rate of low-dose oestrogen therapy in metastatic breast cancer. We are pursuing two paths. To improve long-term antihormone therapy, we are investigating the value of long-term vascular endothelial growth factor receptor (VEGFR)2 inhibitors [28] to block residual oestrogen or selective oestrogen receptor modulator induced VEGF secretion [29]. The recent report that VEGF creates drug resistance to tamoxifen [30] implies that dual long-term adjuvant treatment with tamoxifen and a VEFGR2 inhibitor will have potential clinical merit. However, the key to success, we believe, is the use of low-dose VEGFR2 inhibitor with the adjuvant antihormone to avoid toxicity during long-term therapy.

To improve the value of low-dose oestrogen therapy treatment after exhaustive antihormonal therapy, we believe that the real question is why do 70% of tumours in the clinic not respond to oestrogen induced apoptosis? We have developed cell lines that either respond rapidly or have a delayed response to oestrogen. Using this approach, we have examined the inhibitor of glutathione synthesis buthionine sulfoximine, which has previously been evaluated in the clinic to improve responses to chemotherapy [31]. In preliminary studies, buthionine sulfoximine dramatically enhanced the response of refractory antihormone resistant cells to the early apoptotic actions of oestrogen.

We suggest that there is now a clinical opportunity to use our proposed clinical trial [6,32] design that employs a yet to be determined 12-week course of low-dose oestradiol therapy to treat patients after exhaustive antihormonal therapy. A succession of combined antisurvival agents could potentially improve response rates to well above the 30% rate in metastatic breast cancer rendered refractory by exhaustive antihormonal therapy. The novel test platform is rapid and has tumour response as the end-point. We believe that new combinations of agents could subsequently be employed in much larger trials without oestrogen once its apoptotic efficacy is established.

In closing, it is gratifying that the story of oestrogen action through the oestrogen receptor has continued to offer surprises in each decade since Haddow's report in 1944 [1]. By looking back, we have been able to plan a way forward to benefit patients.

Competing interests

The authors declare that they have no competing interests.

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Jordan, V.C.

A Century of Deciphering the Control Mechanisms of Sex Steroid Action in Breast and Prostate Cancer: The Origins of Targeted Therapy and Chemoprevention

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Abstract

The origins of the story to decipher the mechanisms that control the growth of sex hormone–dependent cancers started more than 100 years ago. Clinical observations of the apparently random responsiveness of breast cancer to endocrine ablation (hormonal withdrawal) provoked scientific inquiries in the laboratory that resulted in the development of effective strategies for targeting therapy to the estrogen receptor (ER; or androgen receptor in the case of prostate cancer), the development of antihormonal treatments that dramatically enhanced patient survival, and the first successful testing of agents to reduce the risk of developing any cancer. Most importantly, elucidating the receptor-mediated mechanisms of sex steroid–dependent growth and the clinical success of antihormones has had broad implication in medicinal chemistry with the synthesis of new selective hormone receptor modulators for numerous clinical applications. Indeed, the successful translational research on the ER was the catalyst for the current strategy for developing targeted therapies to the tumor and the start of "individualized medicine." During the past 50 years, ideas about the value of antihormones translated effectively from the laboratory to improve clinical care, improve national survival rates, and significantly reduced the burden of cancer. [Cancer Res 2009;69(4):1243–54]

Beginnings at the Dawn of the 20th Century

Schinzinger (1) is credited with suggesting that oophorectomy could be used to treat breast cancer; however, this suggestion did not seem to have been adopted. In contrast, the report by Beaston (2) that oophorectomy could initiate a regression of metastatic breast cancer in two premenopausal women was a landmark achievement. Although it is often stated that Beaston's work was empirical clinical research, the rationale to conduct an oophorectomy was, in fact, an example of early translational research. Beaston was aware of the essential role of removing the ovary in maximizing milk production in cows. He reasoned there was potentially some factor that traveled in the blood supply to the breast as there was no known connection through the nerves. Interestingly enough, he also conducted laboratory experiments in rabbits before his clinical experiment, so the work was bench-to-bedside (2). By 1900, Stanley Boyd (3) had assembled the results of all the available clinical cases of oophorectomy to treat breast cancer in Great Britain in perhaps the first "clinical trial." Boyd concluded that only one-third of metastatic breast tumors responded to oophorectomy. This clinical result and overall response rate has remained the same to this day.

Unfortunately, responses were of limited duration and enthusiasm waned that this approach was the answer to cancer treatment. The approach of endocrine ablation was only relevant to breast cancer (and subsequently prostate cancer; ref. 4), thus, the approach was only effective in a small subset of all cancer types. At the dawn of the 20th Century, there was no understanding of the endocrine system or hormones. Nevertheless, laboratory studies started to decipher the biological control mechanisms responsible for the clinical observations.

Links between Sex Steroids and Cancer

The trend in breast cancer research in the early years of the 20th century was to use inbred strains of mice to study the growth and incidence of spontaneous mammary cancer. Lathrop and Loeb (5) found that before age 3 months was the optimal time for oophorectomy to prevent the development of mammary cancer, but obviously, this knowledge could not be translated to the clinical setting; who would one treat? The mechanism was also unknown until Allen and Doisy (6), using an ovariectomized mouse vaginal cornification assay, showed that a principle, that they called estrogen (identified as estrone, the principal steroid), was present in ovarian follicular fluid. Their major advance set the scene for the subsequent breakthroughs in molecular endocrinology and therapeutics in the latter half of the 20th century (Fig. 1).

The idea that breast cancer might be a preventable disease was extended by Professor Antoine Lacassagne (7, 8) who first showed that estrogen could induce mammary tumors in mice. Lacassagne (9) hypothesized, "*If one accepts the consideration of adenocarcinoma of the breast as the consequence of a special hereditary sensibility to the proliferative action of oestrone, one is led to imagine a therapeutic preventive for subjects predisposed by their heredity to this cancer. It would consist—perhaps in the very near future when the knowledge and use of hormones will be better understood—in the suitable use of a hormone, antagonistic or excretory, to prevent the stagnation of oestrone in the ducts of the breasts.*" However, when Lacassagne stated his vision at the annual meeting of the American Association for Cancer Research in Boston in 1936, there were no lead compounds that antagonized estrogen action, but the Allen Doisy mouse assay could be used to study structure activity relationships to find synthetic estrogens. Within a decade, a landmark discovery was to occur in "chemical therapy" that was to expand the treatment of metastatic breast cancer to include postmenopausal women who are, in fact, the majority who develop metastatic disease.

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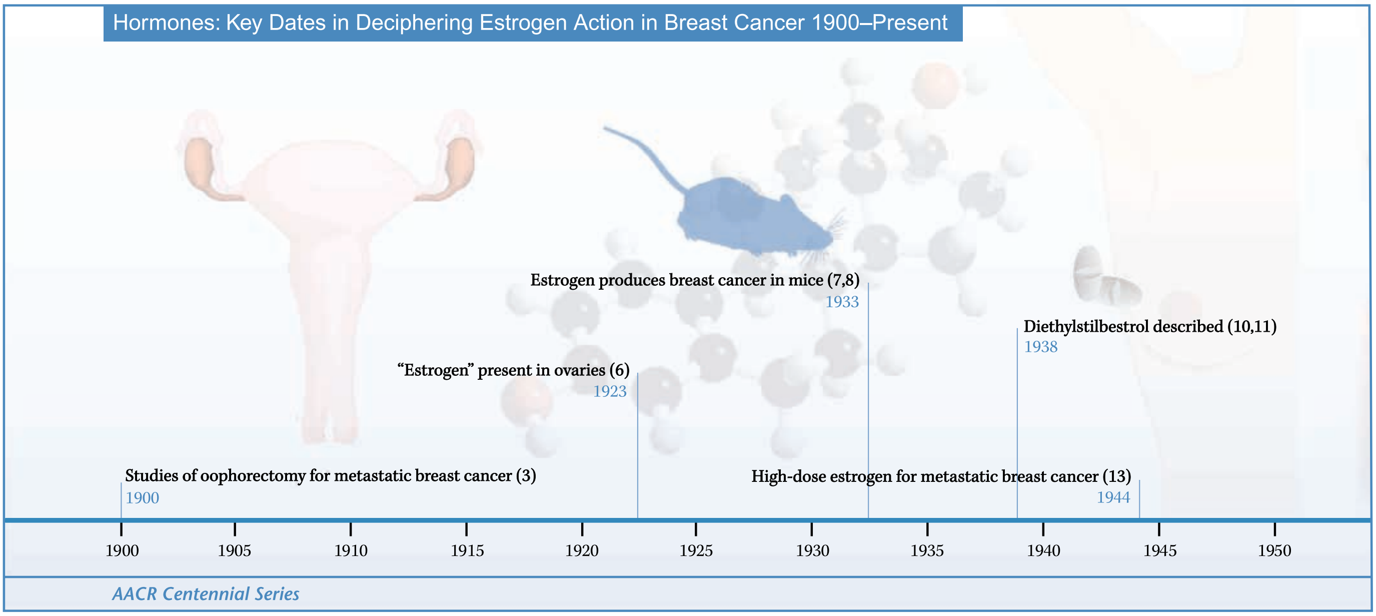

Figure 1. Timeline of the major landmarks in estrogen action and its application for the treatment and prevention of breast cancer.

During the 1930s, there were significant advances in the knowledge of the precise structural requirements for estrogen action in its target tissue, the vagina. Synthetic compounds based on stilbene (10, 11) and triphenylethylene (12) were screened using the Allen Doisy ovariectomized mouse vaginal cornification assay to define compounds with optimal structures and duration of estrogen action. Sir Alexander Haddow found that carcinogenic polycyclic hydrocarbons would cause tumor regression in animals. However, these could not be used to treat humans. The nonsteroidal triphenylethylene-based estrogens had similar structures to polycyclic hydrocarbons and also caused tumor regression in animals. With this clue, Sir Alexander Haddow (13) used the first chemical therapy to treat patients. His results published in 1944 showed that high-dose estrogen therapy was effective in causing tumor regressions in postmenopausal patients with breast cancer and men with prostate cancer. There was, however, no understanding of a mechanism. Indeed he stated in 1970: *"In spite of the extremely limited practicability of such measure [high dose estrogen], the extraordinary extent of tumor regression observed in perhaps 1% of postmenopausal cases has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us"* (14). These experimental data were also an apparent paradox as endocrine ablation to remove estrogens and their precursors was the dogma of the time (15).

In the past 50 years, the progress in deciphering the control mechanisms of estrogen action in breast cancer (and androgen action in prostate cancer), has accelerated with advances in technology and an understanding of cell biology. But progress in research does not travel in straight lines, yet chance observations can create a major breakthrough. This has happened repeatedly in the story of the treatment and prevention of breast cancer.

Conceptual Progress through Scientific Serendipity

It is perhaps relevant to illustrate a few astute observations by scientists that accelerated progress immensely in deciphering the complexities of hormone action and the control of breast cancer growth.

Sir Charles Dodds (11) is credited with the synthesis of the potent synthetic estrogen diethylstilbestrol (Fig. 2) that was subsequently used for the treatment of both prostate cancer and breast cancer, and regrettably was also applied to prevent recurrent abortions (16), which caused an increase in clear cell carcinoma of the vagina in the children (17). During the race to describe the minimal molecular structure that would trigger vaginal cornification in the ovariectomized mouse vagina, controversy erupted in the 1930s over the reproducibility of results concerning the compound anethole. The authors were minimalistic in reporting the synthetic methodology, so replication proved impossible to create the correct biology. Rather the product was correct, but the method used by the *original* authors was not reported accurately and caused dimerization of anethole to an impurity dianethole an estrogen. This active impurity was structurally similar to parallel research endeavors that concluded with the synthesis of the potent estrogen diethylstilbestrol. Thus, the purity of chemicals for testing was critical for successful science.

A similar story was also immensely important in allowing scientists to understand the direct actions of estrogen on the breast cancer cell *in vitro*. The MCF-7 estrogen receptor (ER)-positive breast cancer cell line (18) has been the work horse for the study of estrogen-stimulated growth. However, early examination of MCF-7 cells in the 1970s could not uniformly show estrogen-stimulated growth. Antiestrogens inhibited the apparently constitutive growth of MCF-7 cells, but estradiol did reverse the inhibitory actions of

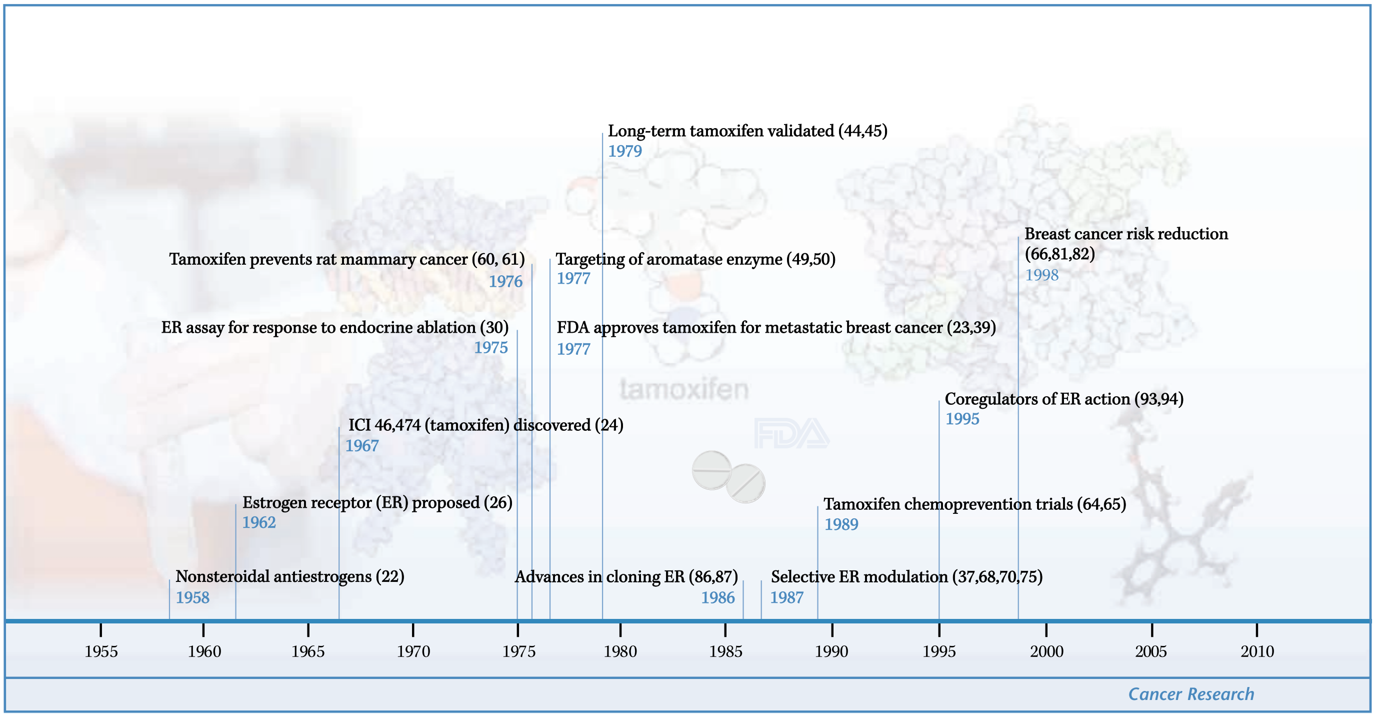

Figure 1. *Continued.*

antiestrogens on growth (19). The mystery deepened when studies *in vitro* could not show estrogen-stimulated growth but MCF-7 cells inoculated into athymic mice would grow into tumors only with estrogen treatment. There was clearly a second factor required for estrogen-stimulated tumor growth *in vivo*! (20).

The astute observations of John and Benita Katzenellenbogen solved the mystery of why estrogen did not stimulate MCF-7 breast cancer cell growth *in vitro*. It seems that all cells had been grown for more than a decade in standard medium containing large concentrations of a pH indicator called phenol red. The Katzenellenbogens realized that the structure of phenol red was similar to nonsteroidal estrogens and removal of the indicator from cell culture media caused cell growth rate to decrease and only then would exogenous estrogen cause growth (21). In other words, the cells were already growing maximally in phenol red containing medium. Subsequent studies revealed that the culprit was, in fact,

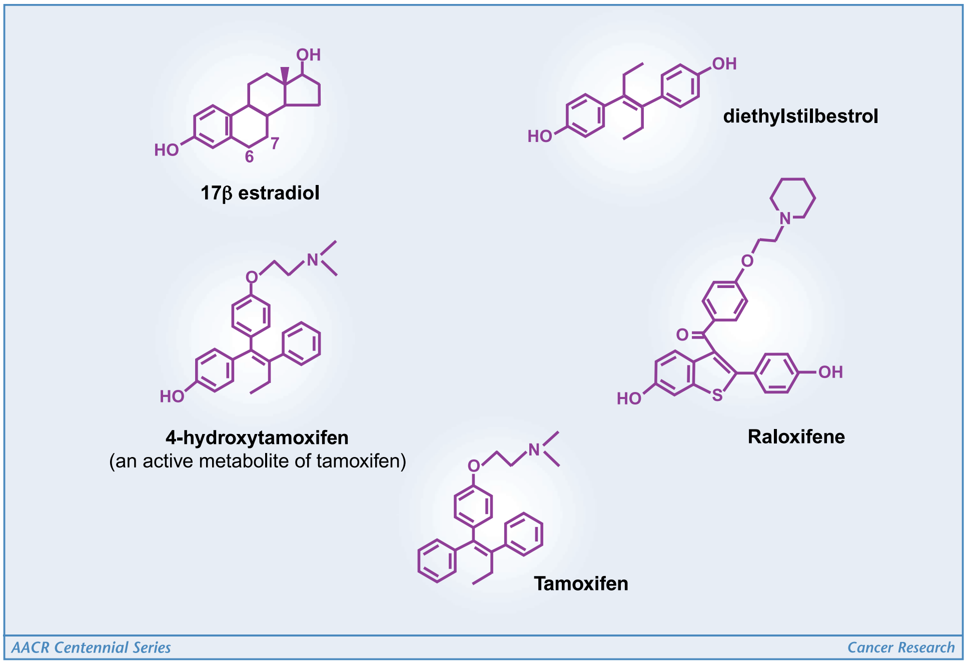
OH
HO
6
7
17β estradiol
OH
HO
diethylstilbestrol
O
N
HO
4-hydroxytamoxifen
(an active metabolite of tamoxifen)
N
O
O
HO
S
OH
Raloxifene
O
N
Tamoxifen
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Figure 2. The structures of estrogens, antiestrogens, and SERMs mentioned in the text. The position 6 and 7 on the estradiol molecule indicate where tritium atoms were inserted to first describe estrogen binding to target tissue (26). The metabolite 4-hydroxytamoxifen (121) is an active metabolite of tamoxifen that has been the standard laboratory antiestrogen and crystallized with the ligand binding domain of the ER (95).

a partially dimerized chemical contaminant of phenol red. This critical technical advance permitted all of the subsequent understanding of the molecular biology of direct estrogen action.

Leonard Lerner (22) was a young research endocrinologist employed by Merrell Dowe to study nonsteroidal estrogen pharmacology. He noticed that the structure of one of the compounds being tested for the control of coronary artery disease was a triphenylethanol similar to the estrogenic triphenylethylenes and he asked to test this chemical as an estrogen. To his surprise, the compound, subsequently renamed MER25 or ethamoxytriphetol, was antiestrogenic in all species tested and had no estrogen-like actions in any animal tests. Lerner (22) had discovered the first nonsteroidal antiestrogen. Although the compound was too toxic and not potent enough for clinical use, Lerner went on to be involved in the discovery of the first triphenylethylene antiestrogen called chloramiphene (MRL41) later to be known as clomiphene (23). Originally, the nonsteroidal antiestrogens were predicted, based on animal studies, to be potent postcoital contraceptives, which in the early 1960s had a huge potential market as “morning after pills.” However, clomiphene did exactly the opposite; it induced ovulation in women (23). Enthusiasm waned and there was general disinterest in this area of research until ICI 46,474, another nonsteroidal antiestrogen discovered in the fertility program of ICI Pharmaceutical Ltd (now AstraZeneca; ref. 24) was reinvented as the first targeted therapy for breast cancer and the first chemopreventive for any cancer (25).

A Target for Treatment and Prevention

The early theory for estrogen action in its target tissues, e.g., uterus, vagina, etc., was that there was chemical transformation between estrone and the less abundant 17β estradiol (Fig. 2) to control the redox potential of the tissue environment. In the late 1950s, Jensen (Fig. 3) and Jacobsen (26) chose another approach at the Ben May Laboratories of the University of Chicago. They synthesized (6, 7) [^{3}H] estradiol (Fig. 2) with very high-specific activity. After its injection into the immature female rats, the unchanged steroid bound to and was retained by the estrogen target tissues: the uterus, vagina, and pituitary gland. In contrast, [^{3}H] estradiol bound to, but was not retained, by nontarget tissues, e.g., muscle, lung, heart. There was clearly a receptor mechanism at play that could be blocked (27) by the coadministration of the first nonsteroidal antiestrogen MER-25 (22).

The mystery of why only about one third of advanced breast cancers responded to either endocrine ablation (3) or high-dose estrogen therapy (15) was solved by the application of basic endocrinology to the practical issue of excluding women with metastatic breast cancer who would not significantly benefit from

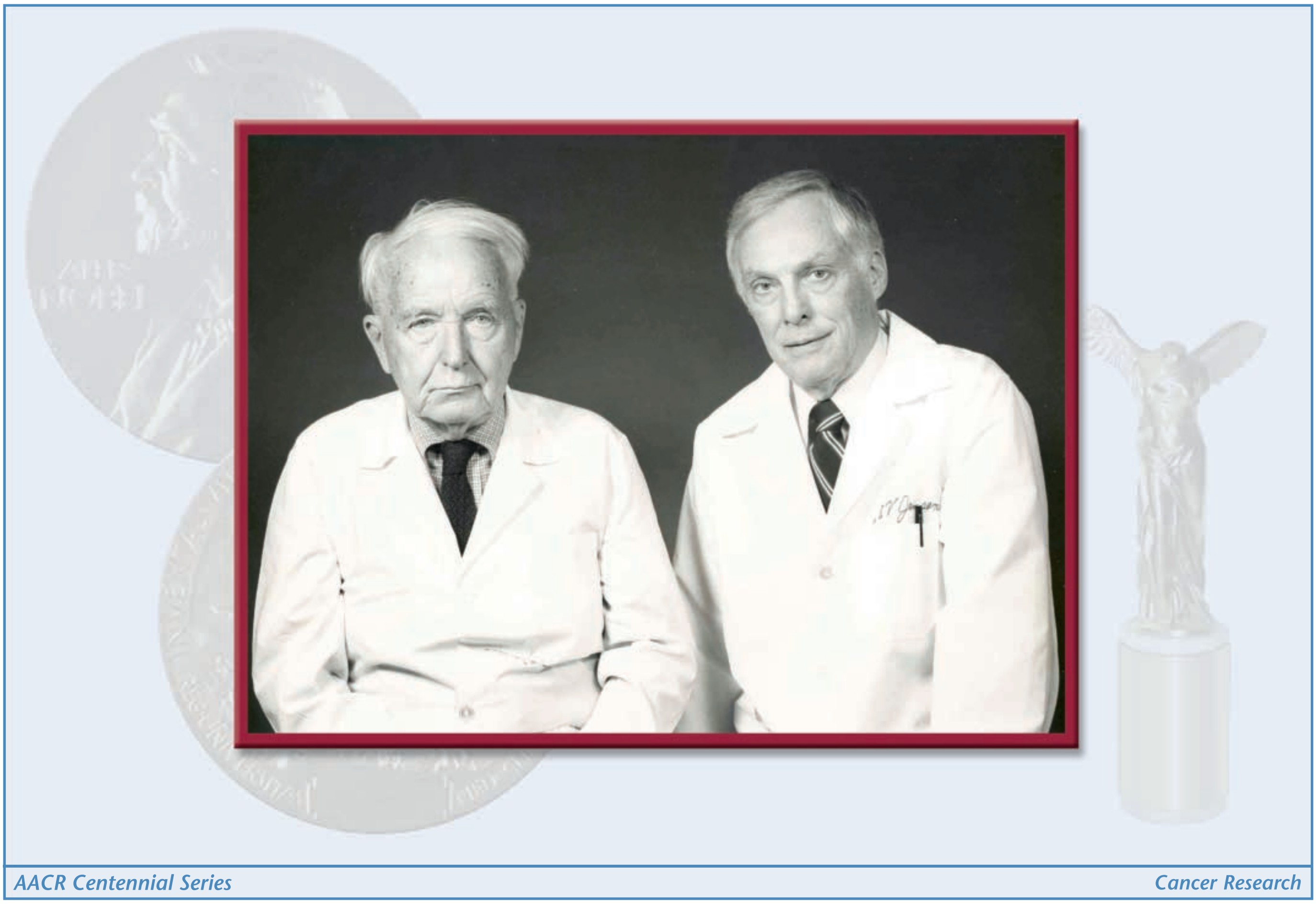


Figure 3. Professor Charles Huggins (*left*) and Elwood Jensen were to receive the Nobel Prize for Physiology and Medicine (1966) and the Lasker Award (2004) for their work on androgen action in cancer and the role of ER in physiology and cancer, respectively.

unnecessary endocrine ablative surgery (oophorectomy, adrenalectomy, or hypophysectomy). The ER was found to be an extractable protein from the rat uterus that would bind [^{3}H] estradiol in the extraction cytosol (28, 29). During the late 1960s, numerous methods were described to identify and quantitative ER levels in tumor biopsies (30) and these data were subsequently correlated with clinical outcomes in metastatic breast cancer (30). Breast tumors without the ER were unlikely to respond to endocrine ablation and therefore should not be treated with this modality. The ER assay was introduced as the standard of care in the mid-1970s to predict endocrine responsiveness to endocrine ablation. It should be stressed that tamoxifen was not available in medical practice until the Food and Drug Administration (FDA) approved this "hormone therapy" in December 1977 for the treatment of metastatic breast cancer in postmenopausal women (23). Indeed, research with the value of the ER assay to predict responsiveness to antiestrogens was unconvincing (23) and the value of adding another hormone therapy to the treatment armamentarium was uncertain. In the 1970s, all hopes in medical oncology were focused on discovering the correct combination of high dose cytotoxic therapies to cure breast cancer much in the same way as both childhood leukemias and Hodgkin's Disease had been cured. This was not to be but translational research took another route; using the ER as a drug target instead of as a predictive test for endocrine ablation (31).

An Unlikely Therapeutic Solution

Professor Paul Ehrlich (1854–1915) established a model for the development of chemical therapies (chemotherapy) to treat infectious disease. A range of chemical therapies would be synthesized to study structure function relationships in appropriate laboratory models that replicated human disease (32). A clinical study would then be performed on the most promising candidate. Ehrlich's pioneering work to develop Salvarsan for the successful treatment of syphilis is a landmark achievement (32). He was, however, unsuccessful in applying the same principles to cancer chemotherapy. Indeed, even as recently as 1970, Sir Alexander Haddow (14) stated that there was unlikely to be a "chemotherapia specifica" like Paul Ehrlich envisioned because cancer was so similar to the tissue of origin. There was also no target or effective tests or models to predict efficacy in cancer treatment before administration to the patient. The key to the successful development of tamoxifen, a failed contraceptive (23), was the application of Ehrlich's principles of developing an effective treatment strategy by using disease specific laboratory models and the use of the tumor ER as a target for drug action (25).

Available laboratory models for the study of the antitumor actions of antiestrogenic drugs were strains of mice with a high incidence of spontaneous mammary tumors (5) or the carcinogen-induced rat mammary carcinoma (33). The mouse models had fallen out of fashion with the discovery of the "Bittner milk factor," a virus that transmits mammary carcinogenesis to subsequent generations through the mother's milk (34). The research community also began to realize that breast cancer was not a viral disease. Nevertheless, the knowledge of mouse mammary carcinogenesis proved to be pivotal for developing precise and targeted promoters to initiate mammary cancer with oncogenes using transgenic mice (35). Another problem with tumor testing of tamoxifen in mice was the unusual observation that tamoxifen, or ICI 46,474 as it was then known, was an estrogen in the mouse (24, 36). This pharmacologic peculiarity became important later with the recognition of selective ER modulation (37). Most importantly, work did not advance quickly in the 1960s and early 1970s, as there was no enthusiasm about introducing a new "hormonal therapy" into clinical practice (25). All early compounds had failed to advance past early clinical studies and only tamoxifen was marketed (23) for the induction of ovulation or the general treatment of late-stage breast cancer in postmenopausal women (38–40).

In the late 1960s, the 7,12-dimethybenz(*a*)anthracene–induced (DMBA) rat mammary carcinoma model (33) was extremely fashionable for research on the endocrinology of rat mammary carcinogenesis (41, 42). However, the parallels with breast cancer are few, as the tumors do not metastasize and are regulated primarily by prolactin secreted by the pituitary gland in direct response to estrogen action (43). Be that as it may, there was no alternative. Therefore, the DMBA rat mammary carcinoma model was adapted to determine the appropriate strategy for the use of antihormonal therapy as an adjuvant. At that time in the mid-1970s, the early adjuvant trials with tamoxifen did not target patients with ER-positive breast cancer and used only short-term (1 year) tamoxifen treatment to avoid premature drug resistance. This duration of tamoxifen that was selected as the antiestrogen only controlled the growth of metastatic breast cancer for about a year (39). The value of short- and long-term (1- or 6-month treatment equivalent to 1 or 6 years of adjuvant treatment in patients) antihormone administration was determined starting treatment 1 month after DMBA administration to 60-day-old Sprague-Dawley rats. Long-term therapy was remarkably effective at controlling the appearance of mammary tumors and was far superior to short term treatment (44, 45). The concepts of targeting the ER and using long-term adjuvant therapy effectively translated through clinical trials to improve national survival rates for breast cancer (46, 47).

Targeting Treatment for Breast Cancer

The early clinical work of Santen (48) established the practical feasibility of using aminogluthemide, an agent that blocks both adrenal steroidogenesis and the CYP19 aromatase enzyme to stop conversion of testosterone and androstenedione to estradiol and estrone, respectively. Unfortunately, aminoglutethimide must be given with a natural glucocorticoid; therefore, long-term therapy is not a practical possibility. Brodie and coworkers (49, 50) advanced knowledge of the specific targeting of the CYP19 aromatase enzyme with the identification and subsequent development of 4 hydroxyandrostenedione (51) as the first practical suicide inhibitor of the aromatase enzyme (Fig. 4). Incidentally, the pivotal work with both tamoxifen and 4-hydroxyandrostenedione (Figs. 2 and 4) was initiated at the Worcester Foundation for Experimental Biology in Massachusetts in the early 1970s (52). Brodie's contribution eventually became the catalyst to create a whole range of agents (e.g., anastrozole; Fig. 3) targeted to the aromatase enzyme for the treatment of breast cancer in postmenopausal women (53). The clinical application of aromatase inhibitors has reduced the side effects noted with tamoxifen in postmenopausal women such as blood clots and endometrial cancer and there has been a small but significant improvement in disease control for the postmenopausal patient when results are compared with tamoxifen (54, 55).

However, recent research into the pharmacogenetics of tamoxifen has suggested that CYP2D6 enzyme product is important for

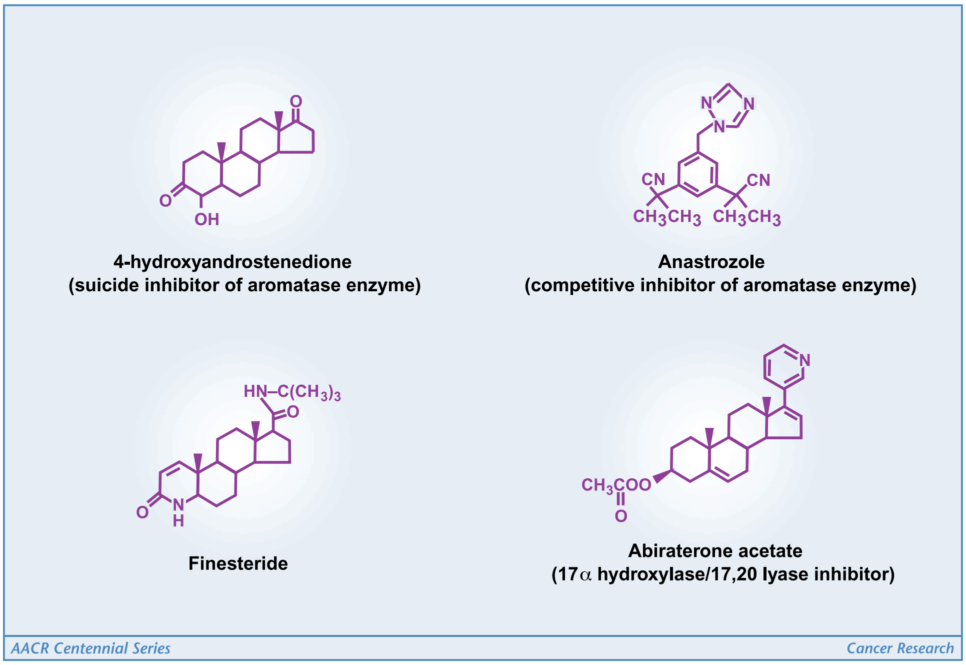


Figure 4. Structures of inhibitors of estrogen and androgen biosynthesis.

metabolism to the active antiestrogen endoxifene (4-hydroxy-N-desmethyltamoxifen; ref. 56), and the use of certain selective serotonin reuptake inhibitors to reduce hot flashes seems to be contraindicated because of drug interaction at the CYP2D6 enzyme (57, 58). Current research is also exploring the hypothesis that a mutated and ineffective CYP2D6 gene product undermines the therapeutic activity of tamoxifen (57, 58). It may be that patients could eventually be selected for optimal effective tamoxifen treatment in cases of ER-positive breast cancer. This would be worthwhile for the chemoprevention of breast cancer. Clearly, the identification of patients for optimal long-term use of tamoxifen should exclude those high-risk women with a mutant CYP2D6 gene who choose to use chemoprevention, as tamoxifen treatment may possibly be suboptimal.

Chemoprevention of Breast Cancer

In the middle of the 1970s, Sporn (59) advanced the concept of the chemoprevention of cancer and strongly advocated this approach as the optimal and clearly most rational way to reduce the burden of cancer. Practical chemoprevention articulated by Lacassagne (9) has its foundations with the finding that tamoxifen prevents DMBA-induced rat mammary carcinogenesis (60, 61). These laboratory findings (45, 60, 61) and the subsequent clinical finding that adjuvant tamoxifen treatment reduces the incidence of contralateral breast cancer (62) prompted Powles (63, 64) to initiate the first exploratory trial to test the worth of tamoxifen to reduce the incidence of breast cancer in high risk women. Although numbers were small, the Powles study did ultimately show the ability of tamoxifen to reduce breast cancer incidence many years after the treatment had stopped (65). In contrast, the large study by Fisher (66, 67) definitively showed the efficacy of tamoxifen to reduce the incidence of ER-positive breast cancer initially and continues to do so after therapy stops in both premenopausal and postmenopausal women at high risk. Tamoxifen became the first medicine approved by the FDA for risk reduction of any cancer. However, concerns based on laboratory findings (68), about the potential of tamoxifen to increase the risk of endometrial cancer in postmenopausal women and the carcinogenic potential of tamoxifen as a hepatocarcinogen (69), demanded that there had to be a better way to reduce the risk of breast cancer as a public health initiative.

The recognition of selective estrogen receptor modulator (SERM) action by nonsteroidal antiestrogens that stimulate some estrogen target tissues but block estrogen-stimulated tumor growth in others, (70) introduced a new dimension into therapeutics and advanced chemoprevention. Raloxifene has its origins as a nonsteroidal antiestrogen for the treatment of breast cancer (71, 72) as LY156758 or keoxifene. The drug failed in that indication (73) and further development was abandoned (74). The discovery that both tamoxifen and keoxifene would maintain bone density in ovariectomized rats (75), block rat mammary carcinogenesis (76), but that keoxifene was less estrogen-like than tamoxifen in the rodent uterus (71) and was less effective in stimulating the growth of endometrial cancer, (77) suggested a new therapeutic strategy (78). Simply stated (79): *"We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids, so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high-risk group to prevent breast cancer."*

Several years later, keoxifene was renamed raloxifene (Fig. 2) and was shown to maintain bone density in osteoporotic or osteopenic women (80), and simultaneously reduce the incidence of invasive breast cancer without causing an increase in the incidence of endometrial cancer (81). Raloxifene went on to be tested against tamoxifen in the Study of Tamoxifen and Raloxifene trial (82) and was FDA approved both for the treatment and prevention of osteoporosis in postmenopausal women and for the reduction of invasive breast cancer incidence in postmenopausal women at elevated risk. The clinical advances with SERMs-modulating estrogen target tissues has provided exceptional opportunities to treat and prevent multiple diseases. However, for the future it is the study of the molecular events of estrogen action that holds the promise of further breakthroughs in patient care.

Molecular Mechanisms of Estrogen and SERM Action

It is not possible to provide a comprehensive review of the explosion of interest in receptor-mediated molecular mechanisms of action of estrogen, so the reader is referred to significant reviews to appreciate the evolution of the topic (83, 84). What will be presented is an evolving guide to current thinking. There are two ERs called α and β (Figs. 5 and 6). The receptor ERα is the traditional ER (26, 28), but it should be stressed that the development of monoclonal antibodies to ER (85) was the essential step for ERα cloning (86, 87) that provided the clues to discover ERβ (88). The receptor proteins encode on different chromosomes and have homology as members of the steroid receptor superfamily, but there are distinct patterns of distribution and distinct and subtle differences in structure and ligand binding affinity. An additional dimension that may be significant for tissue modulation is the ratio of ERα and ERβ at a target site. A high ERα/ERβ ratio correlates well with very high levels of cellular proliferation, whereas the predominance of functional ERβ over ERα correlates with low levels of proliferation (89, 90). The ratio of ERs in normal and neoplasic breast tissue may be an important factor for the long-term success of chemoprevention with SERMs. There is, as a result, much interest in synthesizing ER subtype specific ligands.

There are functional differences between ERα and ERβ that can be traced to the differences in the Activating Function 1 (AF-1) domain located in the amino terminus of the ER (Fig. 6). The amino acid homology of AF-1 is poorly conserved (only 20%). In contrast, AF-2 region located at the C terminus of the ligand binding domain, differs only by one amino acid: D545 in ERα and N496 in ERβ. Because the AF-1 and AF-2 regions are critical for the interaction with other coregulatory proteins and gene transcription, the structural differences between AF-1 provides a clue about the potential functional differences between ER α and β. Studies using chimeras of ER α and β by switching the AF-1 regions show that this region contributes to the cell and promoter specific differences in transcriptional activity. In general, SERMs can partially activate engineered genes regulated by an estrogen response element through ERα but not ERβ (91, 92). In contrast, 4-hydroxytamoxifen and raloxifene can stimulate activating protein-1–regulated reporter genes with both ERα and ERβ in a cell-dependent fashion.

The simple model for estrogen action, with either ERα or ERβ controlling estrogen-regulated events, has now evolved into a fascinating mix of protein partners that have the potential to modulate gene transcription (Fig. 5). It is more than a decade since the first steroid receptor coactivator was first described (93). Now dozens of coactivator molecules are known, and also corepressor molecules exist to prevent the gene transcription by unliganded receptors (94).

It is reasonable to ask how does the ligand program the receptor complex to interact with other proteins? X-ray crystallography of the ligand binding domains of the ER liganded with either estrogens or antiestrogens show the potential of ligands to promote coactivator binding or prevent coactivator binding based on the shape of the estrogen or anti-ER complex (95, 96). Evidence has accumulated that the broad spectrum of ligands that bind to

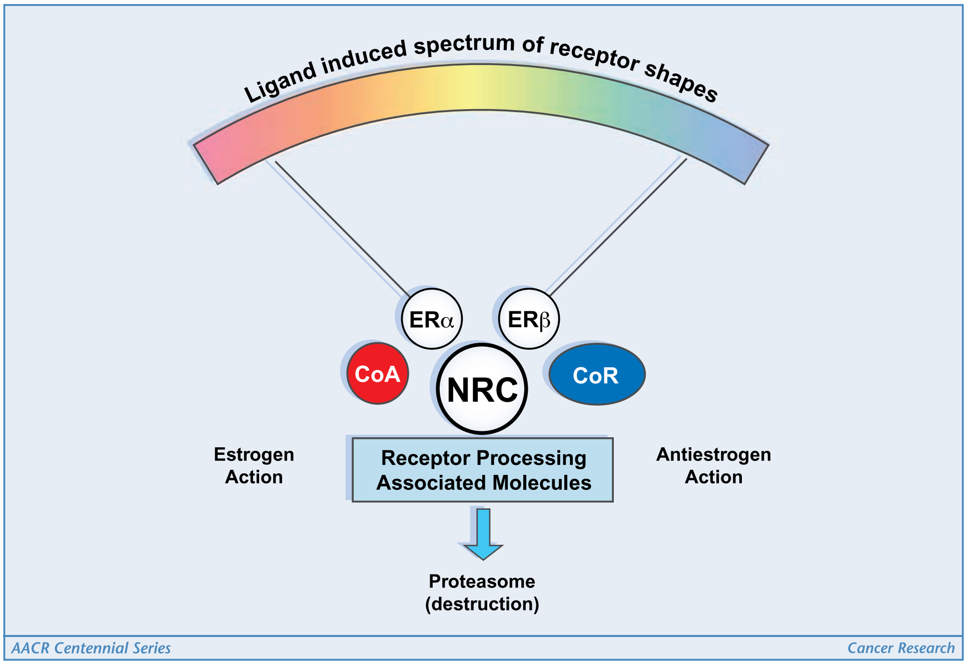


Figure 5. Molecular mechanisms of estrogens, antiestrogens, and SERMs in estrogen target tissues. The nuclear receptor complex (*NRC*) that results from ligand binding to either ERα or ERβ can interact with either coactivators (*CoA*) or corepressors (*CoR*) to initiate estrogenic or antiestrogenic responses, respectively. The activation of transcription at a promoter site of an estrogen responsive gene occurs through the binding of the complex that is cyclically destroyed through the proteosome and then the reassembling of a new complex.

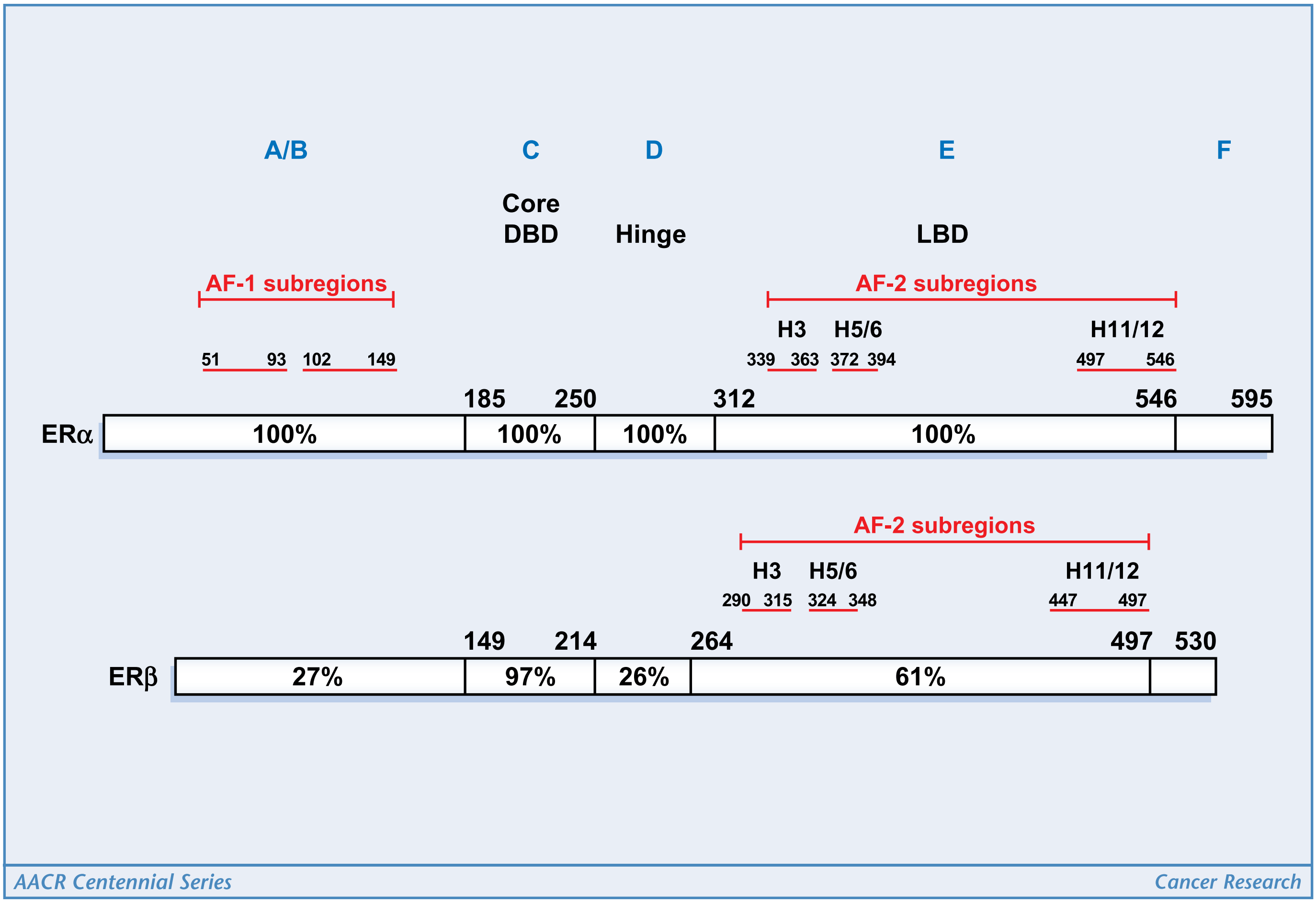


Figure 6. A comparison of the percent homology of the domains of ERs α and β abbreviations: DNA binding domain (*DBD*), ligand binding domain (*LBD*), activating functions (*AF*).

the ER can create a broad range of ER complexes that are either fully estrogenic or antiestrogenic at a particular target site (97). Thus, a mechanistic model of estrogen action and antiestrogen action (Fig. 5) has emerged based on the shape of the ligand that programs the complex to adopt a particular shape that ultimately interacts with coactivators or corepressors in target cells to determine the estrogenic or antiestrogenic response, respectively.

Not surprisingly, the coactivator model of steroid hormone action has now become enhanced into multiple layers of complexity thereby amplifying the molecular mechanisms of modulation (98).The ER complex with its core coactivator (e.g., SRC3) positions itself in the promoter region of an ER responsive gene and attracts associated molecules that engages RNApolII to start transcription. However, the complex of associated molecules also acetylates or deacetylates histones on DNA, thereby regulating the exposure of DNA to modulate transcription. Additionally, associated molecules are recruited to the receptor complex that are members of a family of enzymes that ubiquitinylate proteins in the complex for destruction. Estrogen action is therefore a dynamic process of complex assembly and destruction at the target gene (99).

The complicated modulation of estrogen action at individual target sites is challenging to comprehend but provides opportunities to develop new targeted treatments for sex steroids.

Current Insights into Sex Steroid Modulation

The accumulated knowledge about modulating the ER complex through coregulators interacting at AF-2 and AF-1 create new opportunities for novel drug discovery. The target site modulation of the ER with SERMs has been expanded to the androgen receptor (AR) with selective AR modulators (SARM; refs. 100, 101). existing nonsteroidal SARMs are being used to define tissue specific gene expression that will lead to clinically useful selective anabolic therapies without stimulating the prostate (102).

Studies of the molecular pharmacology of selective nuclear receptor modulators are focused on the relationship between the external shape of the ligand receptor complex and coregulator binding at AF-2 (103, 104). Combinatorial phage display can identify external regions of the receptor complex to map SARM action or create peptide antagonists that will block coactivator binding with potential as new therapies for prostate cancer. Indeed, this approach is now being extended to orphan nuclear receptors that do not need a small ligand for gene regulation (105). Progress with defining cofactors to study the biology of estrogen-related receptor α (ERR-α) is an important advance with significance for new targeted therapeutic agents. The recent description of the role of ERR-α in angiogenesis of ER-negative tumors (106) is a potential practical application of this work.

Posttranslational modifications of sex steroid receptors at AF-1 through phosphorylation cascades have their origins from the cell surface growth factor receptors (107, 108) This knowledge has a potential application to understand the molecular biology of antihormone resistance. However, our evolving knowledge of antihormonal drug resistance has important therapeutic consequences.

Drug Resistance to SERMs

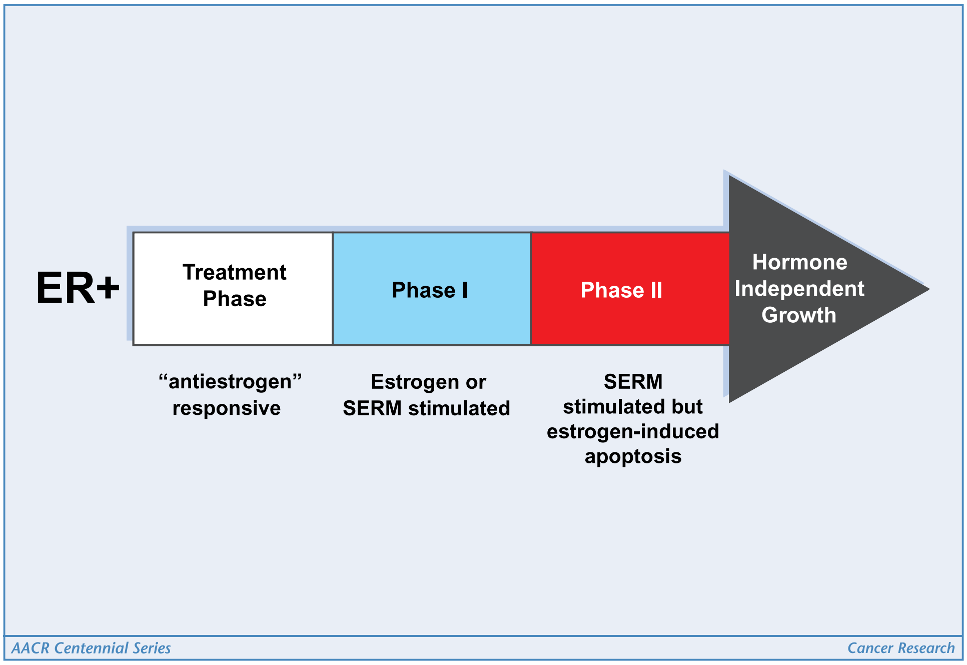
The acceptance of the concept of long-term antihormonal therapy to target, treat, and prevent breast cancer (25) raised the specter of drug resistance to SERMs and SARMs. However, the early models of SERM resistance did not reflect the majority of clinical experience. The natural laboratory models of antihormone resistance caused stimulation of tumor growth during a year of therapy (109), and therefore, reflected drug resistance in patients with metastatic breast cancer who are only treated successfully for a year. The early laboratory models of drug resistance did not replicate clinical experience with adjuvant therapy for 5 years. Remarkably, drug resistance evolves (Fig. 7) and the survival signaling pathways in tamoxifen-resistant tumors becomes reorganized so that instead of estrogen being a survival signal, physiologic estrogen now inhibits tumor growth (110). This discovery provides an invaluable insight into the evolution of drug resistance to SERMS and prompted the reclassification of the process through phase I (SERM/estrogen stimulated) to phase II (SERM-stimulated/estrogen-inhibited growth; ref. 111).

This model would also explain the earlier observations (13) why high-dose estrogen therapy was only effective as a treatment for breast cancer in women many years after the menopause. Natural estrogen deprivation had occurred. The process is accelerated and enhanced, however, in patients treated long term with SERMs or aromatase inhibitors so that only low doses of estrogen are necessary to cause experimental tumors to regress. The new knowledge of the apoptotic action of estrogen (or androgen—see next section) could potentially lead to the discovery of a precise apoptotic trigger initiated naturally by steroid hormone receptors (111). Discovery of this apoptotic trigger might result in an application that targets critical survival signals with new drugs.

Parallel Path of the Prostate

Charles Huggins (Fig. 3; ref. 112) resurrected the use of endocrine ablation for the treatment hormone-dependent breast cancers. His focus, however, was the regulation of the growth of the prostate gland and the application of that knowledge for the treatment of prostate cancer (4). He received the Nobel Prize for Physiology and Medicine in 1966. The process for deciphering the molecular mechanisms of androgen action in its target tissues and prostate cancer has tended to lag behind the pathfinder estrogen. Nevertheless, the basic model for the regulation of nuclear hormone receptor action is consistent but the details of androgen action are distinctly different than estrogen action, which in turn created novel therapeutic opportunities to stop the biosynthesis of each active steroidal agent. The similarities and differences in the molecular actions of estrogen and androgen action are illustrated in Fig. 8. The two significant differences (yet similarities) in the biosynthetic pathways between estrogens and androgens are as follows: (*a*) the aromatization of the A ring of testosterone to create the high-affinity ER binding ligand 17β estradiol in women. This bioactivation led to the development of aromatase inhibitors to block estrogen synthesis (50); and (*b*) the reduction of testosterone to the high-affinity AR binding ligand dihydroxytestosterone in men. This knowledge led to the development of the 5α reductase inhibitor finesteride (Fig. 4) that was tested successfully for risk reduction for prostate cancer in men (113). Unfortunately, as yet, finesteride has failed to advance for use as a chemopreventive for prostate cancer because of overstated concerns about the accelerated development of potentially more aggressive prostate cancers in those men who did not have tumorigenesis prevented. In contrast, aromatase

Figure 7. The evolution of resistance to selective ER modulators (SERMS: tamoxifen or raloxifene) long-term therapy.


ER+
Treatment Phase
Phase I
Phase II
Hormone Independent Growth
"antiestrogen" responsive
Estrogen or SERM stimulated
SERM stimulated but estrogen-induced apoptosis
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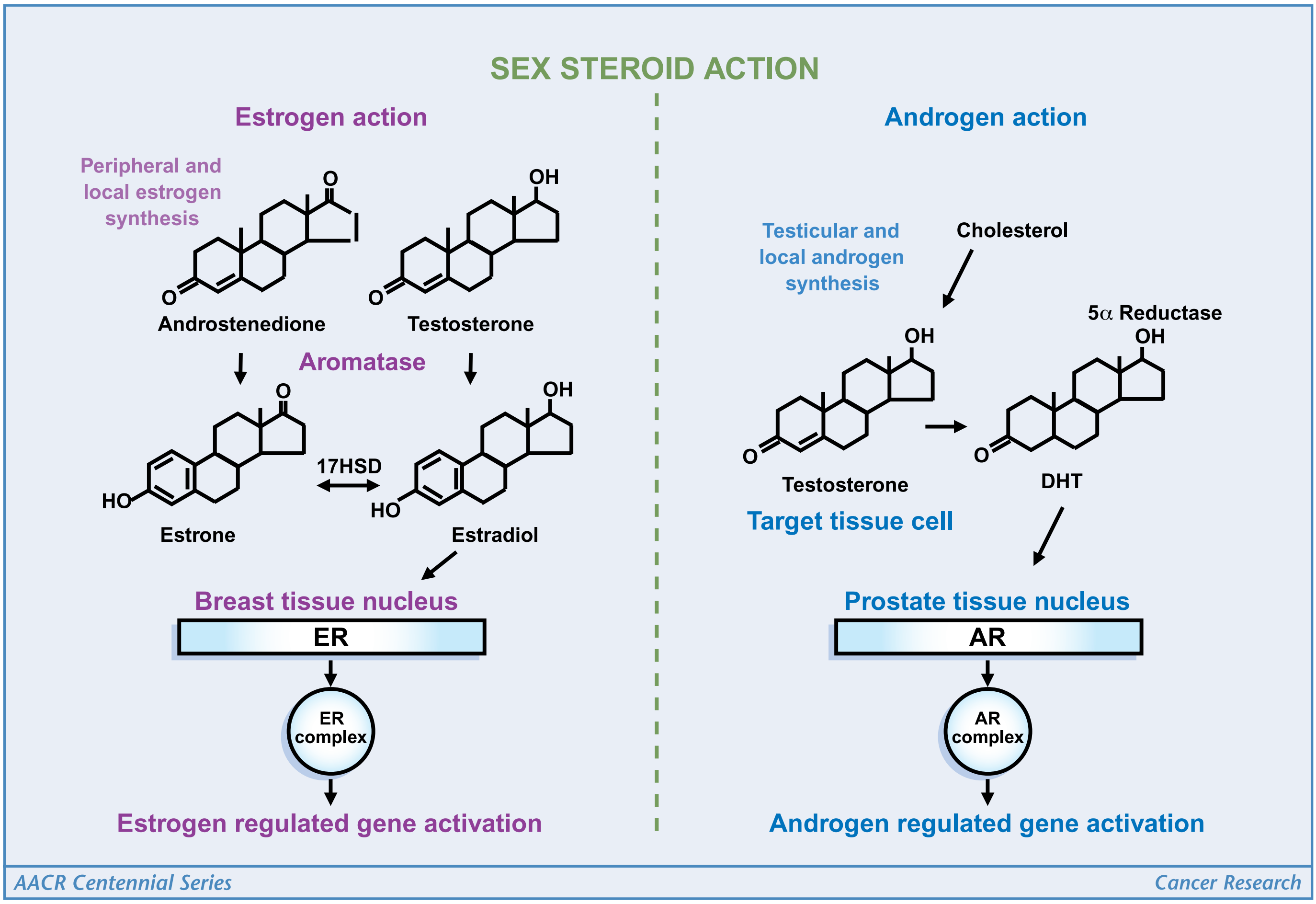
SEX STEROID ACTION
Estrogen action
Peripheral and local estrogen synthesis
Androstenedione
Testosterone
Aromatase
17HSD
HO
Estrone
Estradiol
Breast tissue nucleus
ER
ER complex
Estrogen regulated gene activation
Androgen action
Testicular and local androgen synthesis
Cholesterol
5α Reductase
Testosterone
DHT
Target tissue cell
Prostate tissue nucleus
AR
AR complex
Androgen regulated gene activation
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Figure 8. Comparison of the molecular mechanisms of estrogens and androgens in their respective target tissues. The transformations of the respective steroids are necessary for high binding affinity for their receptors, but the activation from prohormones occurs in different tissue sites relative to their target.

inhibitors have advanced to test their worth as chemopreventive agents (53).

A range of antiandrogenic drugs that competitively block the AR are available in clinical practice (114). Drug resistance to antiandrogen therapy parallels antiestrogen drug resistance (115), and following long-term antihormonal therapy with antiandrogens, androgen induces apoptosis in antiandrogen-resistant prostate cancer cells (116). Recent research has identified high local levels of androgen production as a major form of antihormonal drug resistance (117). As a result, a new therapeutic approach is the development of an inhibitor of androgens biosynthesis from cholesterol (Fig. 8) by blocking 17 hydroxylase/17,20 lyase. A promising compound abiraterone acetate (Fig. 4) is currently being evaluated in clinical trials (118). However, there is also a need to coadminister glucocorticoids so long term therapy must be monitored carefully.

The Successful Evolution of Targeted Antihormonal Therapy in the 20th Century and Beyond

The identification of the ER and subsequently the AR as the conduit for hormone-mediated development and growth in breast and prostate cancer, respectively, has had a profound effect on the approach to the treatment and prevention of cancers. These hormone-mediating molecules have proved to be the pathfinders for the development of targeted therapies that transformed the approach to cancer treatment away from the nonspecific cytotoxic chemotherapy approach during the 1950s to 1990s. As a result, there is current enthusiasm about the promise of individualized medicine and tumor-specific therapeutics (25, 119).

The effect of antihormonal therapy for breast cancer has been profound with improvements in patient survival, a menu of medicines is now available to suit individual patient needs and there is a decrease in national mortality rates in numerous countries (47). Additionally, there are now two SERMS (tamoxifen and raloxifene) available to reduce the incidence of breast cancer (67, 82). But progress in our understanding and application of SERMs is more than chemoprevention. The SERM concept (70) has spread to develop tissue-selective drugs for all members of the hormone receptor superfamily (25, 120). An enormous interest in developing selective glucocorticoid receptor modulators, selective progesterone receptor modulators, SARMs, and even agents to treat rheumatoid arthritis is an ongoing therapeutic outcome of translational research for the chemoprevention of breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Potential of L-buthionine sulfoximine to enhance the apoptotic action of estradiol to reverse acquired antihormonal resistance in metastatic breast cancer☆

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ABSTRACT

L-Buthionine sulfoximine (BSO) is a potent inhibitor of glutathione biosynthesis and studies have shown that it is capable of enhancing the apoptotic effects of several chemotherapeutic agents. Previous studies have shown that long-term antihormonal therapy leads to acquired drug resistance and that estrogen, which is normally a survival signal, is a potent apoptotic agent in these resistant cells. Interestingly, we have developed an antihormone-resistant breast cancer cell line, MCF-7:2A, which is resistant to estrogen-induced apoptosis but has elevated levels of glutathione. In the present study, we examined whether BSO is capable of sensitizing antihormone-resistant MCF-7:2A cells to estrogen-induced apoptosis. Our results showed that treatment of MCF-7:2A cells with 1 nM E2 plus 100 μM BSO combination for 1 week reduced the growth of these cells by almost 80–90% whereas the individual treatments had no significant effect on growth. TUNEL and 4′,6-diamidino-2-phenylindole (DAPI) staining showed that the inhibitory effect of the combination treatment was due to apoptosis. Our data indicates that glutathione participates in retarding apoptosis in antihormone-resistant human breast cancer cells and that depletion of this molecule by BSO may be critical in predisposing resistant cells to estrogen-induced apoptosis.

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1. Introduction

Breast cancer continues to be the most common malignancy affecting women. Although great strides have been made in the treatment and cure of early stage breast cancer, metastatic breast cancer remains incurable resulting in 40,000 deaths per year in the United States alone [1]. Approximately two-thirds of all breast cancers contain the estrogen receptor (ER) and/or progesterone receptor (PgR) and are termed hormonally sensitive disease. A significant proportion of these hormonally sensitive breast cancers are dependent upon estrogenic stimulation for survival and growth [2].

Historically, various techniques employing estrogen deprivation have been utilized to exploit this feature in the treatment of hormonally sensitive breast cancers. Until recently, tamoxifen has been considered to be the hormonal therapy of choice for the treatment of ER-positive breast cancers [3]. Now, survival benefits have been demonstrated for the third generation aromatase inhibitors [4] and the pure anti-estrogen, fulvestrant, that causes degradation of the ER [5].

The use of exhaustive anti-estrogen therapies has consequences for the tumor [6]. With continued long-term estrogen deprivation, these initially hormonally sensitive breast cancer cells become sequentially resistant to further anti-estrogen therapy [7–9], indicating that they develop sophisticated survival mechanisms to sustain growth in estrogen deprived environments (Fig. 1). Jordan and colleagues have demonstrated that when estrogen receptor positive breast cancer cells are grown and maintained in long-term estrogen deprived (LTED) environments, they can ultimately develop enhanced responsiveness to greatly diminished levels of estrogen [6,7,10]. These pre-clinical animal models show that initially, ER expressing tumors are stimulated by estrogen and respond appropriately to tamoxifen with tumor regression. However, with continued exposure to tamoxifen, the tumors become resistant and re-grow [9]. Additionally, treatment of these LTED tumors with post-menopausal levels of estrogen inhibits tumor growth as well as causes regression of established tamoxifen resistant tumors [7,8,11,12] (Fig. 1).

Clinical data supports the use of estrogen to treat hormonally sensitive breast cancers. In the past, pharmacologic doses of estrogen were a commonly employed therapy that resulted in durable responses with regression of disease [13] with as high as 40% response rate as first-line treatment in patients with hormonally sensitive breast cancer with metastatic disease [3] and approximately 31% (44% clinical benefit rate) in patients heavily pre-treated with previous endocrine therapies [14]. Long-term survival data for

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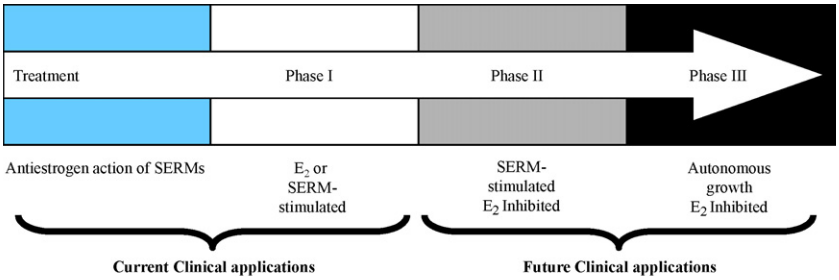
Treatment
Phase I
Phase II
Phase III
Antiestrogen action of SERMs
E_2 or SERM-stimulated
SERM-stimulated E_2 Inhibited
Autonomous growth E_2 Inhibited
Current Clinical applications
Future Clinical applications

Fig. 1. Evolution of drug resistance to selective estrogen receptor modulations (SERMs). Acquired resistance occurs during long-term treatment with a SERM and is evidenced by SERM-stimulated breast tumor growth. Tumors also continue to exploit estrogen for growth when the SERM is stopped, so a dual signal transduction process develops. The aromatase inhibitors prevent tumor growth in SERM-resistant disease and fulvestrant that destroys the ER is also effective. This phase of drug resistance is referred to as phase I resistance. Continued exposure to a SERM results in continued SERM-stimulated growth, but eventually autonomous growth occurs that is unresponsive to fulvestrant or aromatase inhibitors. The event that distinguishes phase I from phase II acquired resistance is a remarkable switching mechanism that now causes apoptosis, rather than growth, with physiologic levels of estrogen.

pharmacologic estrogen treatment in the patients treated as first-line therapy for hormonally sensitive metastatic breast cancer has yielded a statistically significant 5-year survival benefit in favor of estrogen when compared to tamoxifen, 35% and 16% respectively. This clinical data is consistent with the pre-clinical models of Jordan and colleagues that show that after exhaustive anti-hormonal treatment, estrogen treatment produces tumor apoptosis and rapid tumor regression [8,9].

Therefore, we have hypothesized that treatment with a defined course of estrogen in post-menopausal women with ER-positive metastatic breast cancer whose disease has progressed after initial response to sequential anti-estrogen therapies, will result in clinical responses and may potentially reverse hormonally refractory disease, resulting in additional clinical benefit with further endocrine treatment such as an aromatase inhibitor, in this heavily endocrine pre-treated population. We are currently evaluating the optimal dose of daily estradiol therapy to reverse antihormonal resistance [6] but the goal is to enhance the estradiol-induced apoptotic response.

Increased intracellular glutathione has long been associated with tumor cell resistance to various cytotoxic agents. Studies have shown that L-buthionine sulfoximine (BSO) (Fig. 2), a potent inhibitor of glutathione biosynthesis [15], sensitizes tumor cells to apoptosis induced by standard chemotherapeutic drugs *in vitro* and *in vivo* [16,17]. We previously reported the development of a long-term estrogen deprived breast cancer cell line, MCF-7:2A [18], which appeared to be resistant to estradiol-induced apoptosis but expressed elevated levels of glutathione. We believe that the combination of BSO and estradiol could possibly be used to improve the efficacy of estradiol as an apoptotic agent if glutathione depletion is fundamental to tumor cell survival. Our goal is to address the hypothesis that by altering glutathione levels, we may be able to enhance estrogen-induced apoptosis and have employed BSO as our agent of choice.

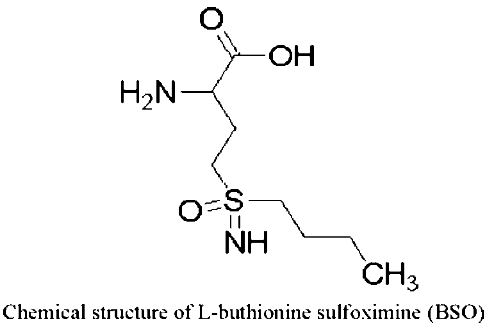

Fig. 2. Chemical structure of L-buthionine sulfoximine.

In the current study, we investigated the *in vitro* effect of the combination of BSO and estradiol (E2) on MCF-7:2A cell viability in relation to apoptosis. We found that BSO or E2, as individual treatments, did not significantly alter the viability of MCF-7:2A cells nor induced apoptosis. However, the combined treatment of BSO and E2 depleted glutathione content and induced significant apoptosis in MCF-7:2A cells. In contrast, similar experiments performed in wild-type hormone responsive MCF-7 cells showed no apoptosis or growth inhibition following the combination treatment of BSO and E2. Our data indicates that glutathione participates in retarding apoptosis in antihormone-resistant human breast cancer cells and that depletion of this molecule by BSO may be critical in predisposing resistant cells to E2-induced apoptotic cell death. We suggest that these data may form the basis of improving therapeutic strategies for the treatment of antihormone-resistant ER-positive breast cancer.

2. Materials and methods

2.1. Cell culture and reagents

The MCF-7 human breast cancer cell line was obtained from Dr. Dean Edwards (University of Texas, San Antonio, TX) and was maintained in phenol red RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1× non-essential amino acids and bovine insulin at 6 ng/mL. The clonal cell line, MCF-7:2A [18], was derived by growing MCF-7 cells in estrogen-free media for more than 1 year, followed by two rounds of limiting dilution cloning. These cells were grown in phenol red-free RPMI 1640 medium supplemented with 10% 4× dextran-coated, charcoal-treated FBS (SFS). All reagents for cell culture were obtained from Invitrogen. BSO and 17beta-estradiol (E2) were from Sigma.

2.2. Cell proliferation

Prior to the start of the cell growth assay, parental MCF-7 cells were grown in estrogen-free RPMI media containing 10% SFS for 3 days. This procedure was performed in order to remove any endogenous estrogen from the serum. On the day of the experiment, MCF-7 and MCF-7:2A cells were seeded in estrogen-free RPMI media containing 10% SFS at a density of 5×10^5 cells per 15-cm dish. After 24 h, cells were treated with nothing (control), 10^{-9} M E2, increasing concentrations of BSO (10 μM to 2.5 mM) either alone or combined with 10^{-9} M E2 for 1 week with retreatment on alternate days. At the indicated time point, the DNA content of the cells was determined as previously described [8] using a Fluorescent DNA Quantitation kit (Bio-Rad). For each analysis, six replicate wells were used, and at least three independent experiments were performed.

2.3. TUNEL staining for apoptosis

Apoptosis was determined by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay using an *in situ* cell death detection kit, POD (Roche Molecular Biochemicals), according to the manufacturer's instructions. Briefly, fixed cells were washed, permeabilized, and then incubated with 50 μL of terminal deoxynucleotidyl transferase end-labeling cocktail for 60 min at 37 °C in a humidified atmosphere in the dark. For signal conversion, slides were incubated with 50 μL of converter-POD (anti-fluorescein antibody conjugated with horse-radish peroxidase) for 30 min at 37 °C, rinsed with PBS, and then incubated with 50 μL of DAB substrate solution for 10 min at 25 °C. The slides were then rinsed with PBS, mounted under glass coverslips, and analyzed under a light microscope (Inverted Nikon TE300).

2.4. 4′,6-Diamidino-2-phenylindole (DAPI) staining for apoptosis

MCF-7:2A cells were grown (overnight) in RPMI medium containing 10% dextran-coated charcoal stripped fetal bovine serum (SFS) and then treated with ethanol vehicle (i.e., control), 1 nM estradiol, 100 μM BSO, or BSO + E2 for 72 h. The cells were then washed in PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, and washed again in PBS. Cells were then treated with 1 μg/mL of DAPI (Sigma Chemical Co.) for 30 min, washed again with PBS for 5 min, and treated with 50 μL of VectaShield (Vector Laboratories, Burlingame, CA). Stained nuclei were visualized and photographed using a Zeiss fluorescence microscope (Provis AX70; Olympus Optical Co., Japan). Apoptotic cells were morphologically defined by cytoplasmic and nuclear shrinkage and by chromatin condensation or fragmentation.

2.5. Glutathione assay

Total cellular glutathione was measured using the Total Glutathione Colorimetric microplate assay Kit (Oxford Biomedical Research), according to the manufacture's protocol. Cells were plated at 0.5×10^6/well of a six-well plate and allowed to recover overnight. After appropriate treatments, cells were washed in PBS and then lysed in 100–150 μL of buffer (100 mM $NaPO_4$, 1 mM EDTA, pH 7.5) containing 0.1% Triton X-100 and frozen at −80 °C until analysis. To measure total glutathione, proteins were precipitated with sulfosalicylic acid at a final concentration of 1%. Samples were then spun for 10 min in a microcentrifuge to pellet proteins, and supernatant was diluted 1:20 in buffer before being measured. For all measurements, 50-μL triplicates of each sample were used for glutathione determination. The GSH level was obtained by comparing with the GSH standards and represented as nmol/mg of protein.

2.6. Statistical analysis

Statistical analysis was performed using Student's *t*-test, and a *P* value of <0.05 was considered significant. Data are expressed as the mean ± S.E. The mean value was obtained from at least three independent experiments.

3. Results

3.1. Glutathione levels are elevated in estrogen deprived MCF-7:2A breast cancer cells

Previous studies have shown that GSH levels in primary breast tumors are more than twice the levels found in normal breast tissue, and levels in lymph node metastases are more than four times the levels in normal breast tissue [19]. Recently, we reported the development of an estrogen deprived breast cancer cell line MCF-7:2A that is resistant to estrogen-induced apoptosis and expresses high levels of the glutathione synthetase gene GSS. To determine whether GSH levels were elevated in our apoptosis-resistant MCF-7:2A breast cancer cell line glutathione assays were performed on these cells. Fig. 3A shows that MCF-7:2A cells had significantly higher levels of GSH at 24, 48, and 72 h (11.9–15.8 nmol/mg protein) compared to wild-type MCF-7 cells (7.8–7.6 nmol/mg protein) and this trend continued up to day 7 (data not show). We next examined whether the GSH synthesis inhibitor BSO was capable of suppressing GSH levels in these cells. Fig. 3B shows that treatment with 100 μM of BSO for 48 h suppressed GSH levels by ∼55% in MCF-7 cells and by ∼75% in MCF-7:2A cells. Longer treatment with BSO (>48 h) yielded similar levels of inhibition (data not shown). These results indicate a possible link between elevated GSH levels and resistance to estrogen-induced apoptosis and they suggest that suppression of GSH by BSO has the ability to reverse the resistant phenotype of the MCF-7:2A cells.

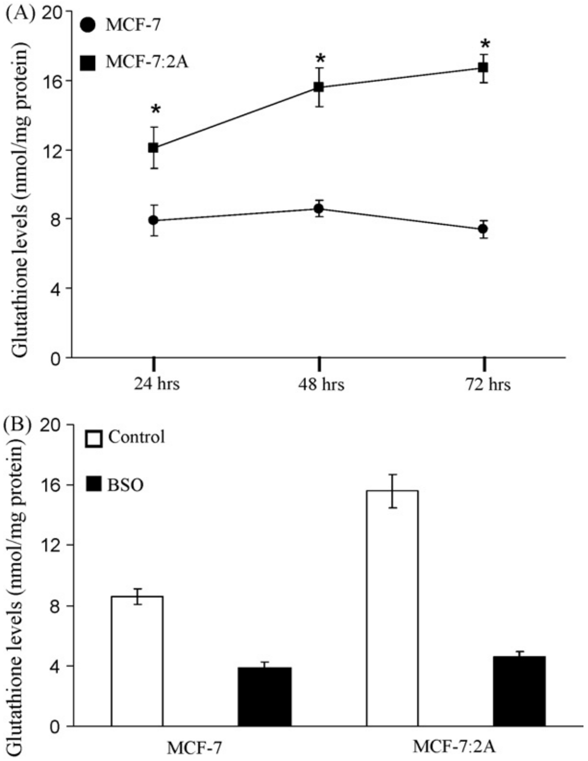

Fig. 3. Intracellular glutathione levels in wild-type MCF-7 cells and antihormone-resistant MCF-7:2A breast cancer cells. (A) Cells were seeded at 2×10^6 cells per 100 mm culture plates in estrogen-free media and total cellular glutathione was measured over a 72-h time period using a glutathione colorimetric assay kit, as described in Section 2. *$P < .0001$, with respect to MCF-7 cells. (B) BSO reduces glutathione levels in MCF-7 and MCF-7:2A cells. For experiment, cells were treated with 100 μM BSO for 48 h and levels of glutathione were measured as described in Section 2. Bars ± S.E.

3.2. Glutathione suppression by BSO sensitizes antihormone-resistant MCF-7:2A cells to estrogen-induced apoptosis

We next examined whether depletion of glutathione by BSO has the ability to sensitize MCF-7:2A cells to estrogen-induced apoptosis. Wild-type MCF-7 cells and estrogen deprived MCF-7:2A cells were seeded in estrogen-free media, and after 24 h, were treated with nothing (control), 1 nM estradiol, or 10 μM to 10 mM BSO in the presence or absence of 1 nM estradiol for 7 days. Fig. 4A shows that the growth of MCF-7 cells was stimulated 5-fold over the con-

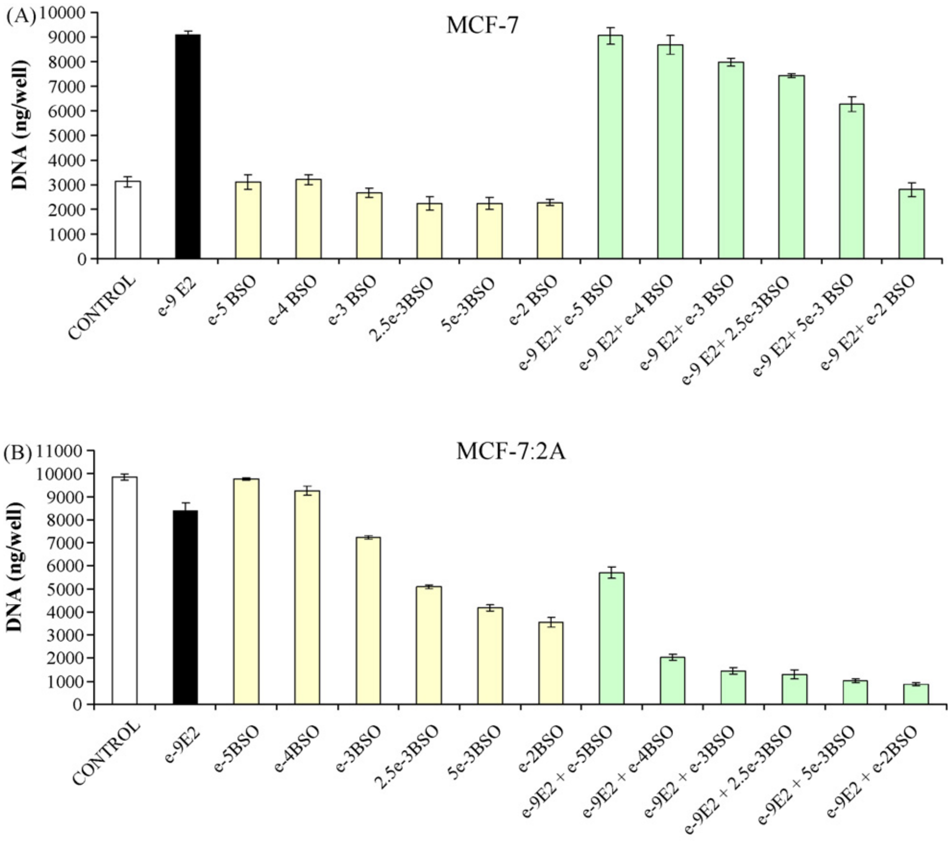

Fig. 4. BSO enhances the growth inhibitory effect of estradiol in antihormone-resistant MCF-7:2A cells. (A) MCF-7 cells were grown in estrogen-free media for 3 days prior to the start of the growth assay. On the day of the experiment, cells were seeded in 24-well plates and after 24 h were treated with various concentrations (10 μM to 10 mM) of BSO in the presence or absence of 1 nM (10^{-9} M) E2 for 7 days. At the indicated time points, cells were harvested and total DNA (ng/well) was quantitated as described in Section 2. (B) MCF-7:2A cells were treated similarly as described above. The data represents the mean of three independent experiments.

trol cells by 1 nM estradiol during the course of the 7-day assay and that treatment with BSO, either alone or in combination with estradiol, did not significantly alter the growth of these cells except at very high concentrations (>1 mM). In contrast, MCF-7:2A cells treated with the combination of BSO and estradiol showed a significant concentration dependent decrease in cell growth relative to cells treated with estradiol or BSO alone (Fig. 4B). It is noteworthy that 100 μM BSO, as a single agent, did not cause growth inhibition of MCF-7:2A cells. However, when combined with 1 nM estradiol the combination caused an 80–90% decrease in growth (Fig. 4B). The cell killing effect of BSO and estradiol was observed as early as 48 h after treatment and persisted over the time course of the experiment with maximum cell death at the 7-day time point. The concentration of BSO used in this study is already known to be clinically achievable without significant side effects [20,21].

Based on the above finding, we next determined whether MCF-7:2A cells underwent apoptotic cell death following BSO plus estradiol treatment. TUNEL assay was performed on cells treated with 100 μM BSO, 1 nM estradiol, or 100 μM BSO plus 1 nM estradiol for 72 h to detect fragmentation of DNA, a characteristic of apoptotic cell death. Fig. 5A shows that the percentage of TUNEL-positive cells significantly increased with the combination of BSO and estradiol but not with estradiol or BSO alone. After treatment with BSO and estradiol (72 h), as many as 53% of cells displayed TUNEL-positive staining, whereas, only 1% of the control cells and 5% of the estradiol treated cells were TUNEL-positive (Fig. 5A). BSO-treated cells looked similar to control cells. As expected, wild-type MCF-7 cells showed very little TUNEL-positive staining in the presence of estradiol alone or BSO plus estradiol combined (data not shown), thus indicating a lack of apoptosis in these cells. DAPI staining of MCF-7:2A cells treated with BSO and estradiol further confirmed that the cells were undergoing apoptosis (Fig. 5B). In addition, phase contrast microscopy of MCF-7:2A cells showed morphological changes associated with apoptosis following BSO and estradiol treatment (Fig. 5C). Overall, these results indicate that BSO, as a single agent, causes neither growth inhibition nor cell death, but is capable of sensitizing antihormone-resistant MCF-7:2A cells to estradiol-induced apoptosis at clinically achievable concentrations.

4. Discussion

In the current study, we investigated whether suppression of the antioxidant glutathione by BSO has the ability to sensitize antihormone-resistant MCF-7:2A breast cancer cells to estradiol-induced apoptosis. Our results showed that glutathione levels were significantly elevated in antihormone-resistant MCF-7:2A breast cancer cells compared to wild-type MCF-7 cells and that the combination treatment of BSO and estradiol caused a dramatic increase in apoptosis whereas the individual treatments had no effect on growth. Noteworthy, the killing effect of BSO and estradiol occurred at clinically achievable concentrations and was observed as early as 48 h. These findings are consistent with previous studies which have shown that the cytotoxicity of a number of chemotherapeutic drugs, including melphalan [22], doxorubicin [23], and bleomycin [24], are significantly enhanced when glutathione is depleted by BSO.

Our laboratory has previously demonstrated that when estrogen receptor positive breast cancer cells are grown and maintained in LTED environments, they can ultimately develop enhanced

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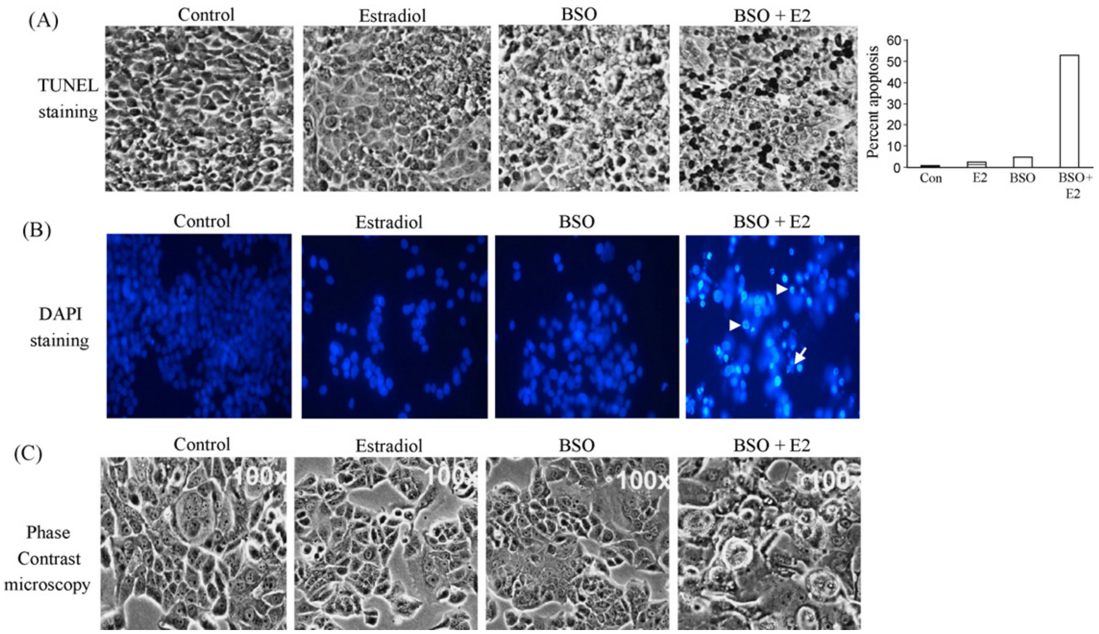

Fig. 5. BSO enhances the apoptotic effect of estradiol in MCF-7:2A breast cancer cells. (A) Cells were treated with 1 nM E2, 100 μM BSO, or 1 nM E2 + 100 μM BSO for 72 h and TUNEL staining for apoptosis was performed as described in Section 2. Slides were photographed through brightfield microscope under 100× magnification. TUNEL-positive cells were stained black (white arrows). Columns (right), mean percentage of apoptotic cells (annexin V-positive cells) from three independent experiments done in triplicate; bars, SEs. (B) Fluorescent microscopic analysis of apoptotic cells stained with 4′,6-diamidino-2-phenylindole (DAPI). MCF-7:2A cells were treated with 1 nM E2, 100 μM BSO, or 1 nM E2 + 100 μM BSO as described above for 72 h. To assess the number of cells undergoing apoptosis, round and/or shrunken nuclei of DAPI-stained cells were counted (white arrows). At least 200 cells per slide were counted by two individuals to control for subjective variability. Experiments were repeated three times with similar results. Representative slides are shown. Scale bars = 50 μM. (C) Phase contrast microscopy of MCF-7:2A cells treated with 1 nM E2, 100 μM BSO, or 1 nM E2 + 100 μM BSO for 72 h.

responsiveness to greatly diminished levels of estrogen [7,9]. These pre-clinical animal models show that initially, estrogen receptor expressing tumors are stimulated by estrogen and respond appropriately to tamoxifen with tumor regression. However, with continued exposure to tamoxifen, the tumors become resistant and re-grow [9]. Additionally, treatment of these LTED tumors with post-menopausal levels of estrogen inhibits tumor growth as well as causes regression of established tamoxifen resistant tumors [7,9,11,12] (Fig. 1). Mechanistic studies indicate that the apoptotic action of estrogen is due to its ability to either activate the fasR/FasL death receptor pathway [11,25] or to disrupt mitochondrial function through activation of the bcl-2 family proteins [7]. The paradoxical action of estrogen in these resistant cells is hypothesized to be due to increased sensitivity to estrogen due to adaptation to estrogen deprivation caused either by tamoxifen or an aromatase inhibitor [26]. It is believed that this “estrogen hypersensitivity” helps to explain the effectiveness of high-dose estrogen in patients with extensive prior endocrine therapy [14].

Interestingly, our present findings indicate that the ability of estradiol to induce apoptosis in antihormone-resistant cells is influenced by the level of glutathione present in the cells. Glutathione levels were elevated ∼1.4- to 1.6-fold in antihormone-resistant MCF-7:2A cells compared to wild-type MCF-7 cells and these cells failed to undergo apoptosis following 1 week of treatment with physiological concentrations of estradiol alone. In the presence of BSO, however, which depleted intracellular glutathione by ∼60–70%, the combination treatment of BSO and estradiol caused a dramatic increase in apoptosis which was observed as early as 48 h with maximum induction observed at day 7. Previous studies have shown that glutathione is an important component of tumor drug resistance [21] and that depletion of intracellular glutathione by BSO significantly enhances the cytotoxicity of many cytotoxic agents, principally alkylating agents [15,20,27] and platinating compounds [16] but also irradiation [28] and anthracyclines [29]. The concentration of BSO used in our study was within the range of 10 μM to 1 mM, which is similar to what has previously been reported in the literature. However, we did observe some toxicity at higher concentrations of BSO (>1 mM) in wild-type MCF-7 and antihormone-resistant MCF-7:2A cells (Fig. 4). It should be noted that BSO, at a clinically achievable concentration of 100 μM, was used for all of our combination experiments with estradiol since this concentration, as an individual treatment, did not significantly alter the growth of MCF-7:2A cells.

Glutathione, a sulfhydryl containing tripeptide, is involved in detoxifying cells from various toxins including chemotherapeutic agents [30,31]. Previous studies have demonstrated a strong correlation between elevated glutathione levels and increased resistance to chemotherapy in cancer cells [32]. This resistance was not limited to the particular chemotherapy agent used to induce resistance, but was also evident when other chemotherapeutic agents were tested for cross-resistance [32]. Additionally, translational studies of *in vitro* cell lines derived from patients with chemorefractory disease were found to have elevated glutathione levels [33]. BSO inhibits γ-glutamylcysteine synthetase (γ-GCS), the rate limiting enzyme in the production of glutathione, thus depleting glutathione levels within the cell [34]. Both, GSH as well as resultant increase in γ-GCS levels as a result of BSO treatment can be monitored peripherally in patients by analysis of peripheral mononuclear cells (PMNs) [35]. BSO also exhibits selectivity in that *in vitro* studies have demonstrated greater depletion of glutathione levels in tumor tissues than sampled normal tissues [30]. Based on its ability to target intracellular glutathione and reverse therapeutic resistance in refractory cancers, BSO is thought to be a potential antineoplastic agent and/or “therapeutic sensitizer” worthy of clinical evaluation.

Early phase clinical trials of BSO at doses resulting in both peripheral and tumor GSH depletion show that BSO can be safely administered to patients with refractory disease. BSO was administered intravenously twice daily either alone or together with chemotherapy to cancer patients whose disease who disease had progressed despite multiple lines of previous chemotherapy

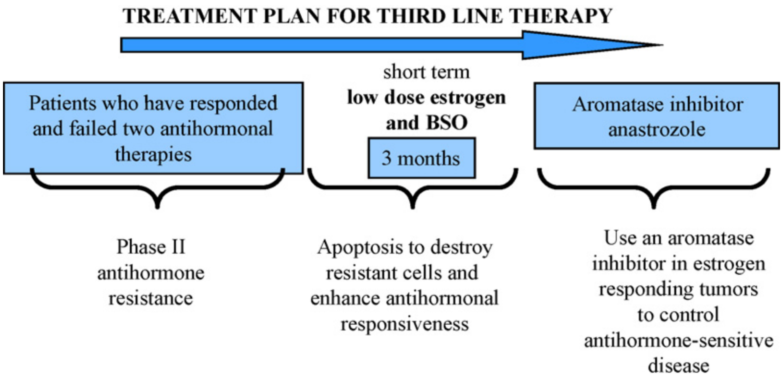
TREATMENT PLAN FOR THIRD LINE THERAPY
Patients who have responded and failed two antihormonal therapies
short term
low dose estrogen and BSO
3 months
Aromatase inhibitor anastrozole
Phase II antihormone resistance
Apoptosis to destroy resistant cells and enhance antihormonal responsiveness
Use an aromatase inhibitor in estrogen responding tumors to control antihormone-sensitive disease

Fig. 6. Clinical protocol to investigate the efficacy of estradiol plus BSO combination treatment to induce apoptosis in long-term endocrine refractory breast cancer. An anticipated treatment plan for third-line endocrine therapy. Patients must have responded and experience treatment failure with two successive antihormone therapies to be eligible for a course of low-dose estradiol combined with BSO therapy for 3 months. The anticipated response rate is 30% and responding patients will be treated with anastrozole until relapse. The overall goal is to increase response rates and maintain patients for longer on antihormone strategies before chemotherapy is required.

[35,36]. In these patients treated with escalating doses of BSO, nausea and vomiting amenable to anti-emetic therapy were the main toxicities. Bone marrow suppression correlating with extent of previous chemotherapy exposure was found to be the rate limiting toxicity in the combination studies. No other significant toxicities were noted. Intracellular glutathione levels measured in PMNs decreased in a linear manner with repeated doses of BSO to a maximum of approximately 10–40% of baseline values [35,36]. When tested in sequential tumor biopsies, glutathione was also found to be depleted to a variable extent in a similarly predictable pattern [36]. Additionally, BSO administration resulted in an initial rapid inhibition of γ-GCS activity followed by γ-GCS recovery during the intervening time between dosings. In fact, γ-GCS levels mirrored peripheral BSO concentrations in patients thus demonstrating targeted delivery of BSO. Clinically, responses to treatment, including complete responses, have been achieved [27,35,36].

In this present study, we demonstrated that glutathione depletion by BSO sensitized antihormone-resistant MCF-7:2A human breast cancer cells to estradiol-induced apoptosis *in vitro*. Taken together, it would be reasonable to incorporate this data into our working translational model for clinical evaluation (Fig. 6). We therefore propose utilizing BSO together with estrogen in patients for a defined therapeutic course in patients with hormonally sensitive metastatic breast cancer whose disease has progressed on prior antihormonal therapies to significantly reduce their disease burden, while potentially reversing resistance to antihormonal therapies. This would then be followed by continuing treatment with an aromatase inhibitor for maintenance of additional clinical benefit for these patients (Fig. 6). Our future goal will be to address this hypothesis in the context of a clinical trial based on these new pre-clinical findings.

Acknowledgments

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Estrogen promotes the survival and pulmonary metastasis of tuberin-null cells

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Lymphangioleiomyomatosis (LAM) is an often fatal disease primarily affecting young women in which tuberin (TSC2)-null cells metastasize to the lungs. The mechanisms underlying the striking female predominance of LAM are unknown. We report here that 17-β-estradiol (E_2) causes a 3- to 5-fold increase in pulmonary metastases in male and female mice, respectively, and a striking increase in circulating tumor cells in mice bearing tuberin-null xenograft tumors. E_2-induced metastasis is associated with activation of p42/44 MAPK and is completely inhibited by treatment with the MEK1/2 inhibitor, CI-1040. In vitro, E_2 inhibits anoikis of tuberin-null cells. Finally, using a bioluminescence approach, we found that E_2 enhances the survival and lung colonization of intravenously injected tuberin-null cells by 3-fold, which is blocked by treatment with CI-1040. Taken together these results reveal a new model for LAM pathogenesis in which activation of MEK-dependent pathways by E_2 leads to pulmonary metastasis via enhanced survival of detached tuberin-null cells.

anoikis | MAPK | lymphangioleiomyomatosis | Bim | Rheb

LAM, the pulmonary manifestation of tuberous sclerosis complex (TSC), affects women almost exclusively (1). LAM affects 30−40% of women with TSC (2, 3). In a Mayo Clinic series, LAM was the third most frequent cause of TSC-related death, after renal disease and brain tumors (4). LAM can also occur in women who do not have germline mutations in *TSC1* or *TSC2* (sporadic LAM). LAM cells from both TSC-LAM and sporadic LAM carry inactivating mutations in both alleles of the *TSC1* or *TSC2* genes (5). The protein products of *TSC1* and *TSC2*, hamartin and tuberin, respectively, form heterodimers (6, 7) that inhibit the small GTPase *R*as *h*omologue *e*nriched in *b*rain (Rheb), via tuberin's highly conserved GTPase activating domain. In its active form, Rheb activates the mammalian target of rapamycin (mTOR) complex 1 (TORC1), which is a key regulator of protein translation, cell size, and cell proliferation (8). Evidence of TORC1 activation, including hyperphosphorylation of ribosomal protein S6, has been observed in tumor specimens from TSC patients and LAM patients (9–11). Independent of its activation of mTOR, Rheb inhibits the activity of B-Raf and C-Raf/Raf-1 kinase, resulting in reduced phosphorylation of p42/44 MAPK (12–14), but the impact of the Raf/MEK/MAPK pathway on disease pathogenesis is undefined.

LAM is characterized pathologically by widespread proliferation of abnormal smooth muscle cells and by cystic changes within the lung parenchyma (1). About 60% of women with the sporadic form of LAM also have renal angiomyolipomas. The presence of *TSC2* mutations in LAM cells and renal angiomyolipoma cells from women with sporadic LAM, but not in normal tissues, has led to the hypothesis that LAM cells spread to the lungs via a metastatic mechanism, despite the fact that LAM cells have a histologically benign appearance (15, 16). Genetic and fluorescent in situ hybridization analyses of recurrent LAM after lung transplantation support this benign metastatic model (16).

The female predominance of LAM, coupled with the genetic data indicating that LAM cells are metastatic, suggests that estrogen may promote the metastasis of tuberin-null cells. Both LAM cells and angiomyolipoma cells express estrogen receptor alpha (17), and there are reports of symptom mitigation in LAM patients after oophorectomy and worsening of symptoms during pregnancy (1). However, the molecular and cellular mechanisms that may underlie an impact of estrogen on the metastasis of LAM cells are not well defined, in part because of the lack of in vivo models that recapitulate the metastatic behavior of LAM cells.

We report here that estrogen promotes the pulmonary metastasis of Tsc2-null ELT3 cells. This enhanced metastasis is associated with elevated levels of circulating tumor cells and with activation of p42/44 MAPK. When Tsc2-null cells are injected intravenously, E_2 enhances their survival and lung colonization, and in vitro, E_2 inhibits anoikis of Tsc2-null cells. In vivo, the MEK inhibitor CI-1040 blocks E_2-induced lung metastasis, decreases circulating tumor cells, and reduces lung colonization. Taken together, these data reveal that the MEK pathway is a critical component of the estrogen-dependent metastatic potential of Tsc2-null cells and lead to a unique model of LAM pathogenesis with therapeutic implications in which E_2 promotes the survival of disseminated LAM cells, thereby facilitating lung colonization and metastasis.

Results

Estrogen Promotes the Pulmonary Metastasis of Tuberin-Deficient ELT3 Cells in Ovariectomized Female Mice and in Male Mice. To study the role of E_2 in the metastasis of Tsc2-null cells, we used ELT3 cells, which were originally derived from a uterine leiomyoma in the Eker rat model of Tsc2 and, similar to LAM cells, express smooth muscle cell markers and estrogen receptor alpha (18, 19). To confirm that ELT3 cells proliferate in response to estrogen stimulation in vitro, cell growth was measured using ^{3}H-thymidine incorporation. E_2 treatment resulted in a significant increase in ^{3}H-thymidine incorporation by 2.8-fold on day 5 ($P = 0.03$, Fig. 1*A*), similar to the findings of Howe *et al.* (19).

ELT3 cells were inoculated s.c. into the flanks of ovariectomized CB17-*SCID* mice, which were supplemented 1 week before with either placebo or E_2 pellets (2.5 mg, 90-day release). Tumors arose in 100% of both estrogen and placebo-treated mice. At post-inoculation week 8, estrogen-treated mice had a mean tumor area

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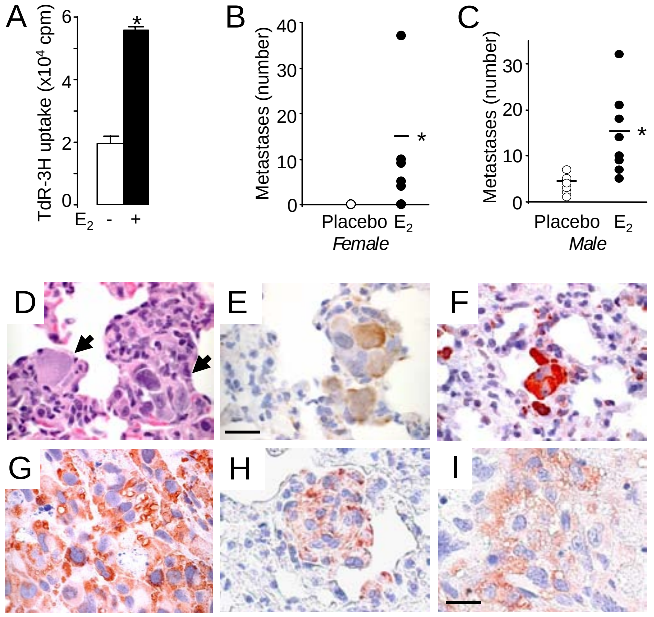

Fig. 1. Estrogen promotes the lung metastasis of tuberin-deficient ELT3 cells in female and male mice. (*A*) The proliferation of ELT3 cells in response to E_2 was measured by ^{3}H-thymidine incorporation after 5 days of growth. (*B–J*) ELT3 cells were injected s.c. into the flanks of female ovariectomized and male *SCID* mice implanted with E_2 ($n = 9$) or placebo ($n = 10$) pellets. (*B*) Lung metastases were scored from E_2 ($n = 9$) or placebo-treated ($n = 10$) mice. (*C*) The number of lung metastases in male mice was scored from placebo ($n = 10$) and E_2-treated ($n = 9$) mice. (*D–I*) Consecutive lung sections containing metastases (arrows) from an E_2-treated female mouse were stained with H&E (*D*), anti-smooth muscle actin (*E*), and anti-phospho-S6 (*F*). (Scale bar, 50 μM.) (*G*) Anti-phospho-S6 immunostain of the primary xenograft tumor of an estrogen-treated female mouse. (*H* and *I*) Phospho-S6 immunoreactivity of a metastasis (*H*) and xenograft tumor (*I*) of an estrogen-treated male mouse. (Scale bar, 20 μM.) *, $P < 0.05$, Student's *t* test.

of 287 ± 43 mm^2, whereas placebo-treated mice had a mean tumor area of 130 ± 20 mm^2 ($P = 0.0035$), consistent with previous findings (19). The proliferative potential of ELT3 cells in vivo was examined using Ki-67 immunoreactivity. The number of Ki-67 positive cells in estrogen-treated tumors was 17% higher than the number in placebo-treated tumors ($P = 0.03$).

Pulmonary metastases were identified in 5 of 9 E_2-treated mice (56%), with an average of 15 metastases/mouse (range 4–37) (Fig. 1*B*). In contrast, only 1 of 9 placebo-treated mice (10%) developed a single metastasis ($P = 0.039$). To determine whether the enhanced metastasis was directly related to tumor size, a subset of placebo-treated mice ($n = 4$) and estrogen-treated mice ($n = 4$) that developed primary tumors at similar size (209 ± 16 and 198 ± 20 mm^2, respectively) was analyzed separately. Three of the estrogen-treated mice developed pulmonary metastases with an average of 6 metastases/mouse, while none of the placebo-treated mice developed metastases.

Next, we inoculated ELT3 cells into male mice. At 8 weeks post-cell inoculation, E_2-treated animals developed tumors that were 2.9-fold larger than those in the placebo-treated animals. As in the female mice, E_2 significantly enhanced the frequency and the number of pulmonary metastases. At 8 weeks post-inoculation, 10 of 10 (100%) of the E_2-treated mice developed metastases, with an average of 14 metastases/mouse (range 5–32). In contrast, 7 of 10 (70%) of the placebo-treated mice developed metastases, with an average of 4 metastases/mouse (range 1–7, $P = 0.013$) (Fig. 1*C*). As expected, the metastatic and primary tumor cells were immunoreactive for smooth muscle actin and phospho-ribosomal protein S6 (Fig. 1 *D–I*).

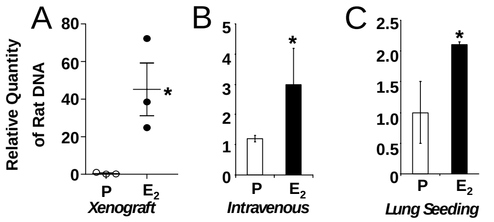

Fig. 2. Estrogen increases circulating tumor cells in mice bearing xenograft tumors and enhances the survival and lung seeding of intravenously injected Tsc2-null cells. (*A*) DNA prepared from the blood of placebo ($n = 3$) and E_2-treated ($n = 3$) mice bearing xenograft tumors of similar size (≈1,000 mm^3) was analyzed by real-time PCR using rat-specific primers to quantitate circulating tumor cells. (*B*) Levels of circulating tumor cell DNA 6 h after i.v. injection of ELT3 cells into placebo ($n = 3$) and E_2-treated ($n = 3$) mice. (*C*) Levels of tumor cell DNA in the lungs 24 h after i.v. injection of ELT3 cells into placebo ($n = 3$) and E_2-treated ($n = 3$) mice. *, $P < 0.05$, Student's *t* test.

Estrogen Increases Circulating Tumor Cell DNA. To determine whether the mechanism of E_2-driven metastasis of ELT3 cells is associated with an increase in survival of ELT3 cells in the circulation, we analyzed blood collected from xenograft mice at 7 weeks post-cell inoculation. Real-time PCR with rat-specific primers was used to measure the relative quantity of tumor cells circulating in the blood. We selected 6 animals (3 placebo, 3 E_2-treated) bearing tumors of similar size (≈1,000 mm^3) for this analysis. The E_2-treated animals had a striking increase in the amount of circulating tumor cell DNA as compared to that in the placebo-treated animals ($P = 0.034$, Fig. 2*A*).

This increased level of circulating tumor cell DNA suggested that E_2 may promote the survival of Tsc2-null cells upon dissemination from the primary tumor site. To test this, we injected 2×10^5 ELT3 cells intravenously and again measured the amount of tumor cell DNA using real-time PCR. E_2 treatment resulted in a 2.5-fold increase in circulating cells 6 h post-injection ($P = 0.047$, Fig. 2*B*). To determine whether this enhanced survival of circulating cells was associated with increased colonization of the lungs, the mice were killed 24 h after injection, and the lungs were analyzed by real-time PCR. E_2 treated mice had a 2-fold increase in the lung seeding of ELT3 cells ($P = 0.039$, Fig. 2*C*).

Estrogen Promotes the Lung Colonization of ELT3 Cells in Vivo. To identify the earliest time points at which estrogen exerts an effect on the survival of intravenously injected Tsc2-null cells, ELT3 cells that stably express luciferase (ELT3-Luc) were intravenously injected. The level of bioluminescence was evaluated using the Xenogen IVIS System. At 1 h post-cell injection, similar levels of bioluminescence were observed in the chest regions of E_2 and placebo-treated mice. By 3 h, the bioluminescence in the chest regions was 2-fold higher in the E_2-treated animals than in the placebo-treated animals, and at 24 h post-cell injection it was 5-fold higher in the E_2-treated animals ($P = 0.043$, Fig. 3 *A* and *B*). After sacrifice, the lungs were dissected and imaged in Petri dishes to confirm that the bioluminescent signals in the chest regions of the living mice were a result of lung colonization (Fig. 3*C*).

Estrogen Activates p42/44 MAPK in ELT3 Cells in Vitro and in Vivo. These results suggested that E_2 promotes the survival of disseminated ELT3 cells. To determine the mechanism of this, we focused on the Raf/MEK/MAPK signaling cascade. This pathway is inhibited in cells lacking TSC2 via Rheb's inhibition of B-Raf and C-Raf/Raf-1 kinase (13, 14). E_2 has been shown to activate p42/44 MAPK in ELT3 cells and in LAM patient-derived cells (11, 20, 21). To confirm that E_2 activates MAPK in ELT3 cells, we treated the cells with 10 nM E_2 and examined the phosphorylation status of p42/44 MAPK by immunoblotting. Within 15 min, E_2 induced the phosphorylation of p42/44 MAPK (Fig. 4*A*). We also found that

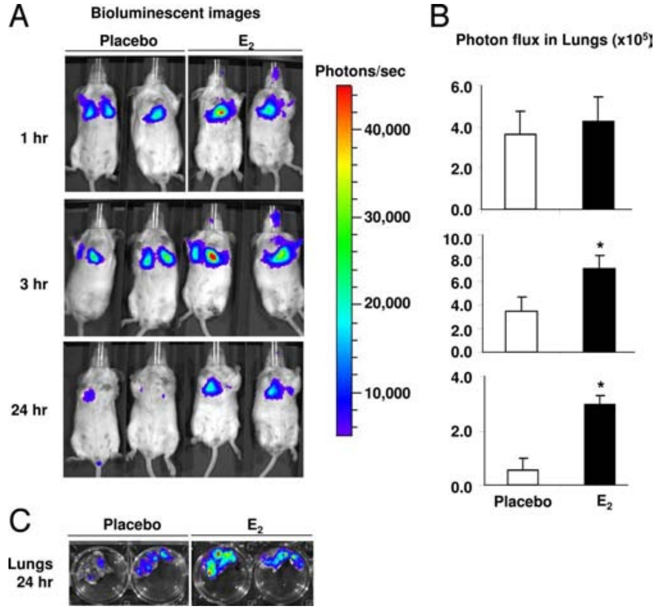

Fig. 3. Estrogen promotes the lung colonization of Tsc2-null ELT3 cells. (*A*) ELT3-luciferase cells were injected intravenously into ovariectomized female placebo ($n = 3$) and E_2-treated ($n = 3$) mice. Lung colonization was measured using bioluminescence at 1, 3, and 24 h after injection. Representative images are shown. (*B*) Total photon flux/second present in the chest regions in placebo ($n = 3$) and E_2-treated ($n = 3$) animals. *, $P < 0.05$, Student's *t* test. (*C*) Lungs were dissected 24 h postcell injection and bioluminescence was imaged in Petri dishes.

E_2-induced phosphorylation of p42/44 MAPK was blocked by the MEK1/2 inhibitor PD98059 (Fig. 4*A*), which is in contrast to the prior work of Finlay *et al.* (20). E_2 is known to rapidly activate C-Raf (22). We hypothesized that E_2 reactivates MAPK via a Rheb-independent pathway in cells lacking tuberin. In a separate experiment, we found that E_2 rapidly (within 2 min) increased the phosphorylation of C-Raf at Ser-338, a site which is closely linked with C-Raf activity (Fig. 4*B*). However, E_2 does not affect mTOR activation as measured by ribosomal protein S6 phosphorylation (Fig. 4*C*). These results suggest that E_2 does not regulate Rheb activity and that the potential of E_2 to impact the Raf/MEK/ERK kinase cascade is Rheb independent. Nuclear translocation of phospho-MAPK was observed within 5 min of E_2 exposure (Fig. 4*D*).

These in vitro findings led us to examine whether E_2 activates p42/44 MAPK in ELT3 cells in vivo. In lungs from E_2-treated animals, nuclear phospho-p42/44 MAPK staining was observed in metastases but not in adjacent normal tissues (Fig. 4 *E* and *F*). In the primary xenograft tumors, the percentage of cells with primarily nuclear phospho-MAPK was significantly higher in the tumors from the E_2-treated animals, compared to the tumors from placebo-treated animals (65% vs. 28%, $P = 0.001$, Fig. 4 *G–I*).

Estrogen Increases the Resistance of ELT3 Cells to Anoikis in Vitro. These in vivo findings suggest that estrogen enhances the survival of circulating tumor cells in a MAPK-dependent manner. Because detached cells normally undergo apoptosis (23–25), a critical first step in cancer progression is the development of resistance to matrix deprivation-induced apoptosis (anoikis) (26, 27). Therefore, to investigate the mechanism of E_2-prolonged survival of ELT3 cells in the circulation, we examined the effect of estrogen on anoikis. ELT3 cells were treated for 24 h with either 10 nM E_2 or control and then plated onto PolyHEMA, which prevents attachment and therefore induces anoikis. Cell lysates were immunoblotted for cleaved caspase-3, which is a measure of apoptosis. E_2 treatment reduced caspase-3 cleavage at 6, 16, and 24 h (Fig. 5*A*). E_2 treatment also significantly reduced DNA fragmentation at 1 and 24 h ($P =$

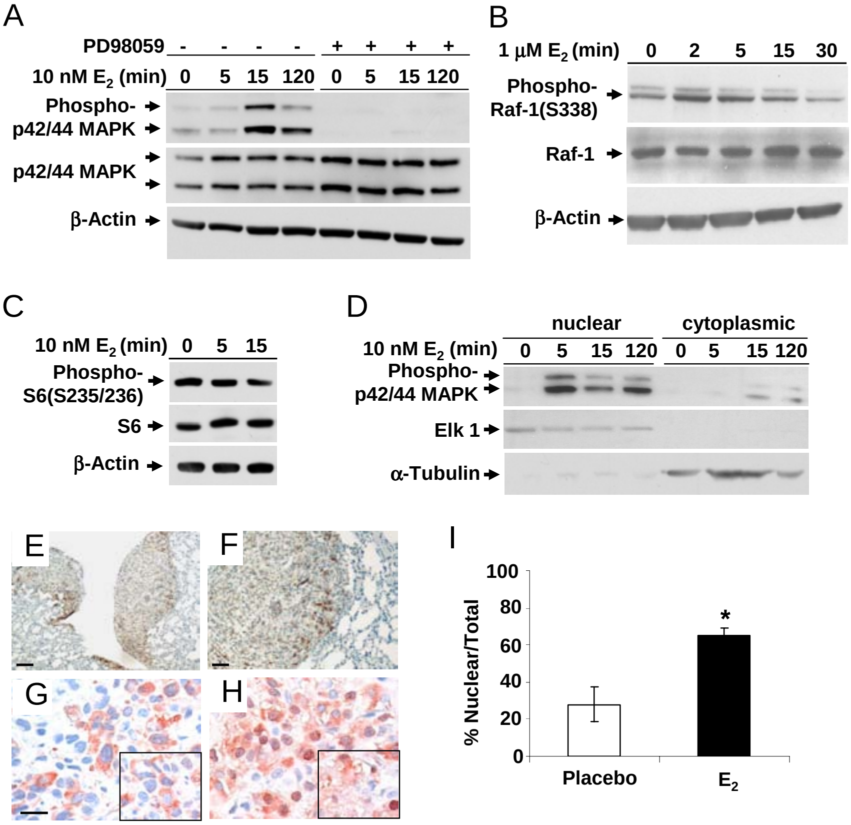

Fig. 4. Estrogen activates p42/44 MAPK in ELT3 cells in vitro and in vivo. (*A*) ELT3 cells were grown in phenol red-free and serum-free media for 24 h and then stimulated with 10 nM E_2 for 0, 5, 15, or 120 min. Levels of phosphorylated p42/44 MAPK and total MAPK were determined by immunoblot analysis. Pretreatment with PD98059 blocked E_2-induced MAPK activation. β-Actin immunoblotting was included as a loading control. (*B*) Levels of phosphorylated C-Raf/Raf-1 and total Raf-1 after E_2 stimulation. (*C*) Levels of phosphorylated S6 after E_2 stimulation. (*D*) The nuclear and cytoplasmic fractions were separated, and levels of phospho-p42/44 MAPK were examined by immunoblot analysis. Anti-ELK1 and anti-α-tubulin were included as loading controls for the nuclear and cytosolic fractions, respectively. (*E* and *F*) Pulmonary metastases from an E_2-treated mouse showed hyperphosphorylation of p42/44 MAPK. (Scale bar, 50 μM and 125 μM.) (*G* and *H*) Phospho-p42/44 MAPK (T202/Y204) immunostaining of primary tumor sections from placebo-treated (*G*) and E_2-treated (*H*) mice. (Scale bar, 20 μM.) (*I*) Percentage of cells with nuclear immunoreactivity of phospho-p42/44 MAPK was scored from 4 random fields per section. *, $P < 0.05$, Student's *t* test.

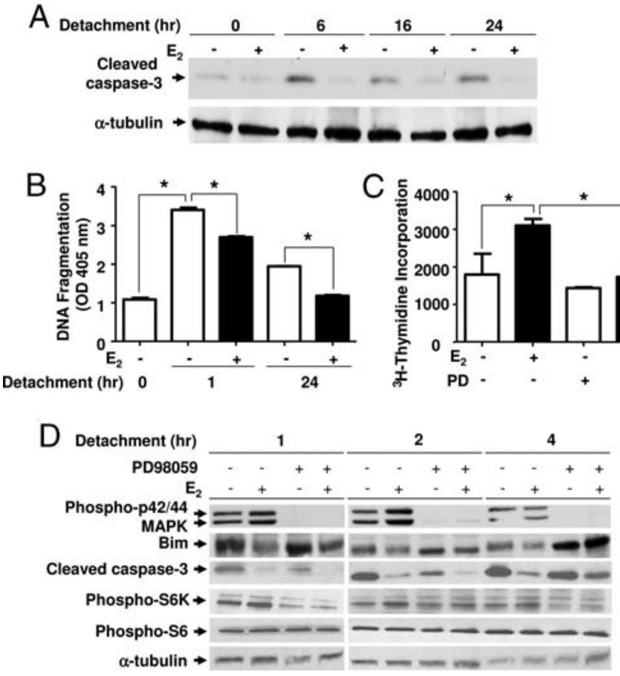

Fig. 5. Estrogen increases the resistance of ELT3 cells to anoikis. ELT3 cells were grown in phenol red-free and serum-free media for 24 h and then treated with 10 nM E_2 for 24 h before culturing on PolyHEMA plates. The MEK1/2 inhibitor PD98059 was begun 15 min before detachment. (*A*) The level of cleaved caspase-3 was determined by immunoblot analysis. α-Tubulin is included as a loading control. (*B*) DNA fragmentation was assessed by ELISA. (*C*) Cell growth was measured by ^{3}H-thymidine incorporation after 24 h of growth on PolyHEMA plates in the presence or absence of E_2, followed by 24 h of growth on adherent plates in the absence of E_2. (*D*) Levels of phospho-p42/44 MAPK, MAPK, Bim, cleaved caspase-3, phospho-S6K, and phospho-S6 were determined by immunoblot analysis. α-Tubulin is included as a loading control. *, $P < 0.05$, Student's *t* test.

0.001 and $P = 0.015$, Fig. 5*B*), which indicates that E_2 inhibits anoikis of Tsc2-null cells.

To confirm further that E_2 promotes the survival of detached cells, ELT3 cells were plated onto PolyHEMA plates for 24 h and replated onto normal tissue culture dishes. Cell growth was measured using ^{3}H-thymidine incorporation. E_2 treatment resulted in a significant increase in ^{3}H-thymidine incorporation 24 h after replating ($P = 0.008$, Figure 5*C*). This E_2-enhanced survival was blocked by treatment with the MEK1/2 inhibitor PD98059 ($P = 0.035$, Fig. 5*C*).

To determine the components that mediate estrogen-enhanced resistance of ELT3 cells to anoikis, we analyzed the proapoptotic protein, Bcl-2 interacting mediator of cell death (Bim), which is known to be a critical activator of anoikis (23). Bim is phosphorylated by protein kinases, including p42/44 MAPK, which leads to rapid proteasomal-mediated degradation and increased cell survival (28). Bim protein level was examined by immunoblotting. We found that estrogen decreased the accumulation of Bim after 1 h in detachment conditions (Fig. 5*D*). Preincubation with the MEK inhibitor PD98059 partially blocked estrogen's inhibition of Bim accumulation and caspase-3 cleavage after 4 h in detachment conditions (Fig. 5*D*). We also examined the phosphorylation of S6K and S6 in detachment conditions and found that the phosphorylation of S6K and S6 did not change with E_2 stimulation. Interestingly, treatment with PD98059 decreased the phosphorylation of S6K 1 h after detachment (Fig. 5*D*).

The MEK1/2 Inhibitor CI-1040 Blocks the Estrogen-Driven Metastasis of ELT3 Cells in Vivo. These in vitro and in vivo results suggest that E_2-induced activation of the MEK/MAPK pathway contributes to the metastatic potential of circulating Tsc2-null ELT3 cells. To determine the effect of inhibiting the MEK/MAPK pathway on the pulmonary metastasis of Tsc2-null cells in vivo, we used the MEK1/2 inhibitor, CI-1040. Beginning 1 day post-subcutaneous inoculation of ELT3 cells, animals, implanted with either placebo or estrogen pellets, were treated with CI-1040 (150 mg/kg day by gavage, twice a day) (29). CI-1040 delayed tumor formation (Fig. 6*A*) and reduced the size of primary tumors by 25% in E_2 animals (Fig. 6*B*), although these data did not reach statistical significance. CI-1040, however, significantly reduced the levels of circulating ELT3 cells in the blood of E_2-treated animals by 84% ($P = 0.042$, Fig. 6*C*). Most strikingly, no lung metastases were detected in mice treated with E_2 plus CI-1040 ($P = 0.046$, Fig. 6 *D* and *E*).

To investigate further the role of MEK/ERK on the survival of ELT3 cells in the circulation, ELT3-luciferase cells were intravenously injected into mice treated with E_2 alone or E_2 plus CI-1040. At 2 h post-cell injection, similar levels of bioluminescence were observed in the chest regions of all mice. At 5 h, the bioluminescence in the chest regions of the E_2 plus CI-1040 treated mice was decreased by 55%, as compared to that in the E_2-treated mice ($P = 0.02$, Fig. 6*F*). After sacrifice at 60 h postcell injection, the bioluminescent signals in the ex vivo lungs of the E_2 plus CI-1040-treated mice were significantly reduced by 96%, as compared to the signals in the E_2-treated animals ($P = 0.0045$, Fig. 6*F*).

Inhibition of mTOR Blocks Estrogen-Induced Pulmonary Metastasis of Tsc2-Null Cells. To determine the role of mTOR signaling pathway in the estrogen-induced metastasis of tuberin-deficient ELT3 cells, the mTORC1 inhibitor RAD001 (4 mg/kg/day by gavage) was administered 5 days per week beginning 1 day post-cell inoculation. RAD001 completely blocked both primary tumor development (Fig. 7*A*) and lung metastasis (Fig. 7*B*) in the presence of estrogen or placebo.

Discussion

LAM is associated with a very unusual disease mechanism: the metastasis of histologically benign TSC1 or TSC2-null cells. LAM has one of the strongest gender predispositions of any extragenital human disease, with a higher female-to-male ratio than even breast cancer. Estrogen receptor alpha is expressed in LAM cells and in angiomyolipoma cells from LAM patients (17), and estrogen has been shown to activate p42/44 MAPK and stimulate the proliferation of Tsc2-null ELT3 cells and TSC2-null angiomyolipoma cells (11). Estrogen has also been shown to enhance liver hemangioma development in Tsc2± mice (30). Despite these findings, the role of estrogen in LAM pathogenesis is not well defined.

We report here that estrogen treatment of both female and male mice bearing Tsc2-null ELT3 xenograft tumors results in an increase in pulmonary metastases. The estrogen-driven metastasis of ELT3 cells was associated with activation of p42/44 MAPK both in vitro and in vivo. Treatment of the mice with the MEK1/2 inhibitor CI-1040 completely blocked the lung metastases in estrogen-treated animals, while causing only a 25% reduction in the size of the primary xenograft tumors, indicating that activation of MEK by E_2 is a critical factor in the metastasis of Tsc2-null cells. In contrast to CI-1040, the mTOR inhibitor RAD001 completely blocked formation of the primary tumor.

Estrogen is known to activate the MAPK pathway (31–34). We speculate that tuberin-null cells may be particularly sensitive to activation of the Raf/MEK/MAPK signaling cascade by estrogen, because at baseline this signaling pathway is inhibited by Rheb, the target of tuberin's GTPase activating protein domain (12–14). Metastasis is a complex process, and there are numerous mechanisms through which estrogen's activation of MEK may enhance the metastasis of Tsc2-null cells. Our in vitro studies revealed that estrogen induces resistance to anoikis in Tsc2-null cells, which suggests that one of these mechanisms involves the survival of detached cells. Consistent with this, we found markedly elevated

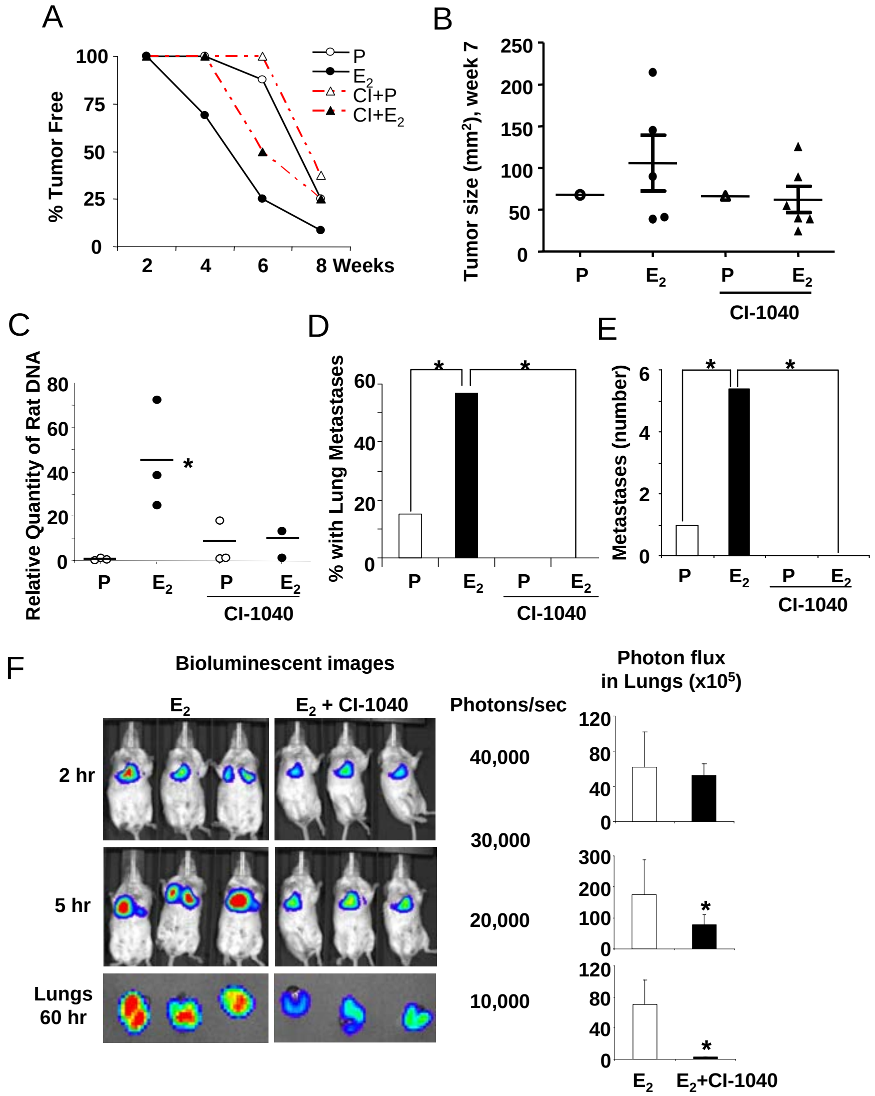

Fig. 6. The MEK1/2 inhibitor CI-1040 blocks the estrogen-driven metastasis of ELT3 cells in vivo. ELT3 cells were injected into female ovariectomized nude mice implanted with estrogen or placebo pellets. Animals were treated with CI-1040 (150 mg/kg/day by gavage, twice a day) starting 1 day post-ELT3 cell inoculation for the xenograft experiments (*A*–*E*), or 2 days before cell inoculation for i.v. injection (*F*). (*A*) Tumor development was recorded as the percentage of tumor-free animals post-cell inoculation. (*B*) The primary tumor area was calculated at 7 weeks post-cell inoculation. (*C*) The level of circulating ELT3 cells was measured from blood samples of xenograft animals using rat-specific qPCR amplification. (*D*) The percentage of mice with lung metastases in the placebo and estrogen-treated groups was compared. (*E*) The number of lung metastases was scored. (*F*) ELT3-luciferase cells were injected intravenously into ovariectomized female E_2-treated ($n = 5$) and CI-1040 plus E_2-treated ($n = 5$) mice. Lung colonization was measured using bioluminescence 2 and 5 h after injection. Total photon flux/second present in the chest regions were quantified and compared between E_2 ($n = 5$) and CI-1040 plus E_2-treated ($n = 5$) animals. Lungs were dissected and imaged 60 h post-cell injection. Total photon flux/second present in ex vivo lungs were quantified and compared between E_2 ($n = 5$) and CI-1040 plus E_2-treated ($n = 5$) animals. *, $P < 0.05$, Student's *t* test.

levels of circulating tumor cells in estrogen-treated mice bearing xenograft tumors. We also found that estrogen treatment enhances the survival of intravenously injected cells in the peripheral blood. These data are of particular interest because circulating LAM cells can be detected in the blood and pleural fluid of women with LAM (35). Our data provide a rationale for the potential use of circulating cells as a quantitative and rapid biomarker of response to targeted therapy in women with LAM.

In addition to promoting the levels of ELT3 cells in the peripheral blood, as measured by real-time RT-PCR using rat-specific primers, estrogen also enhanced the survival of intravenously injected luciferase-expressing ELT3 cells within the lungs. Three hours after injection, there was significantly more bioluminescence in the chest regions of the E_2-treated animals, and by 24 h this difference was even more marked. Importantly, however, 1 h after the i.v. injection of ELT3-luciferase cells, similar levels of bioluminescence were present in the lungs of estrogen-treated and placebo-treated animals, which demonstrates that similar numbers of injected cells reach the lungs. These data suggest that E_2 promotes the survival of Tsc2-null cells within the lungs.

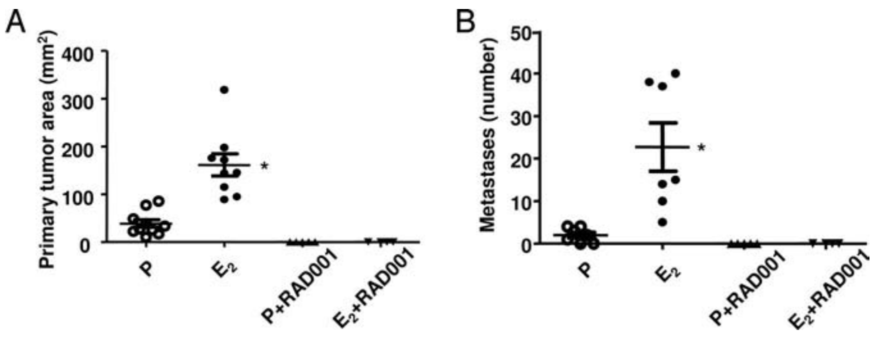

Fig. 7. The mTOR inhibitor RAD001 blocks primary tumor development and estrogen-driven metastasis of ELT3 cells in vivo. ELT3 cells were injected into female ovariectomized nude mice implanted with estrogen or placebo pellets. Animals were treated with RAD001 (4 mg/kg/day by gavage) starting 1 day post-ELT3 cell inoculation. (*A*) The primary tumor area was calculated at 8 weeks post-cell inoculation. (*B*) The number of lung metastases was scored at 8 weeks post-cell inoculation. *, $P < 0.05$, Student's *t* test.

The lack of an in vivo model of LAM has been a significant barrier in LAM research. While not a perfect surrogate, ELT3 cells have important features in common with LAM cells, including loss of Tsc2, activation of mTOR, and expression of estrogen receptor alpha and smooth muscle markers (18, 19). We are optimistic that this model of estrogen-induced metastasis will allow agents to be tested preclinically, thereby facilitating the development of therapies for LAM. Currently the only effective therapy for end-stage LAM is lung transplantation, and many women die while awaiting a donor lung or as a complication of the transplantation. There are multiple nodes that one can target in the estrogen/MEK/MAPK pathway, including inhibition of estrogen production, inhibition of the estrogen receptor, and inhibition of Raf/MEK.

Taken together, our data highlight a unique model for LAM pathogenesis in which activation of MEK by estrogen promotes the survival of detached tuberin-null cells. It will be important to confirm these findings using patient-derived cells, although this will be challenging because of the difficulties in establishing cultures of LAM cells. An alternative would be to measure levels of circulating LAM cells in women receiving hormonal therapy in the context of a clinical trial. If our model is correct, then important effects of estrogen on LAM pathogenesis may occur before the LAM cells reach the lungs and/or within the first hours of their reaching the lungs. Therefore, targeting estrogen signaling may have a major role in the treatment of early-stage LAM and/or in the prevention of LAM in women with TSC.

Methods

ELT-3 cells are Eker rat uterine leiomyoma-derived smooth muscle cells and were used in all in vitro and in vivo studies. For in vivo studies, female ovariectomized CB17-*SCID* mice were implanted with 17-beta estradiol or placebo pellets (2.5 mg, 90-day release) 1 week prior to cell inoculation. For xenograft tumor establishment, 2×10^6 ELT3 cells were bilaterally injected into the rear flanks of the mice. For intravenous injections, 2×10^5 ELT3 or ELT3-Luc cells were injected into the lateral tail vein. Lung metastases were scored from 5-micron H&E-stained sections of each lobe. CI-1040 (150 mg/kg day by gavage, twice per day) or RAD001 (4 mg/kg per day by gavage) was initiated 1 day after cell inoculation. To detect circulating ELT3 cells, 0.5 mL of mouse blood was collected by intraocular bleed, red blood cells were lysed, and genomic DNA was extracted. At death, lungs were dissected for DNA extraction. The assay for rat DNA was adapted from the method described by Walker *et al*. (36). Bioluminescent reporter imaging was performed to monitor the lung seeding of ELT3-Luciferase cells. Ten minutes prior to imaging, animals were injected with luciferin (Xenogen) (120 mg/kg, i.p.). Bioluminescent signals were recorded at indicated times post-cell injection using the Xenogen IVIS System. Total photon flux at the chest regions and from the dissected lungs was analyzed. For anoikis studies, ELT3 cells with or without 10 nM E_2 pretreatment were plated onto poly-hydroxyethyl methacrylate (PolyHEMA) culture dishes. Cell death as a function of DNA fragmentation was detected using Cell Death Detection ELISA kit (Roche Diagnostics). Full methods are available in *SI Text*.

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Resistance to paclitaxel therapy is related with Bcl-2 expression through an estrogen receptor mediated pathway in breast cancer

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Abstract. Taxanes are approved for the treatment of breast cancer that has spread to the lymph nodes, following surgery and doxorubicin containing chemotherapy. Taxanes have improved the survival of breast cancer patients, especially in estrogen receptor (ER) negative population in clinical settings. This time we examined the relationship between chemosensitivity to Taxanes and expresson of ERα in breast cancer cell lines. *In vitro* effects of paclitaxel in 4 ER-positive and 3 ER-negative breast cancer cell lines were investigated by MTT assay. We also investigated members of Bcl-2 family by Western blotting and RT-PCR to clarify their role in paclitaxel resistance both in ER-positive and in ER-negative cells. ER-negative cell lines were more sensitive to paclitaxel than ER-positive cells. ER-negative KPL-4 and ZR-75-30 cells, which were sensitive to paclitaxel, became resistant when they were treated with demethylation agent, 5-aza-2'-deoxycytidine. Analysis of proapoptotic (Bax) and antiapoptotic (Bcl-2) molecules suggested that Bcl-2 is likely to have a role in the resistance of ER-positive cells. Bcl-2 expression was increased in a time-dependent manner after treatment of ER-positive cell lines with estrogen (E2). On the other hand, Bcl-2 was not detected in ER-negative cell lines. However, no significant difference was detected for Bax mRNA levels before and after E2 treatment in ER-positive and negative cell lines. Activation of ER gene expression in ER-negative KPL-4 cells by 5-aza-2'-deoxycytidine resulted in up-regulation of Bcl-2 mRNA. To support our data, we examined paclitaxel sensitivity in ER-negative MDA-MB-231 and ER stable transfectant cells S30 and JM6. This experiment also showed ER-negative cells were sensitive to paclitaxel but ER-positive cells were resistant to it. These results suggest that ER influenced chemosensitivity to paclitaxel through regulation of Bcl-2 family and regulation of the pathway may be crucial to increase the efficacy of taxanes in ER-positive breast cancer.

Introduction

Adjuvant chemotherapy with anthracycline-based regimens has been proven to decrease the risk of relapse and cancer-related mortality in breast cancer patients. The taxanes, paclitaxel and docetaxel, have been successfully incorporated into several adjuvant chemotherapy regimens in recent studies. The available studies reveal that the addition of taxanes after surgery and doxorubicin containing chemotherapy clearly shows a benefit in the adjuvant treatment of breast cancer especially in the lymph node-positive cases. Taxanes act by shifting the dynamic equilibrium between tubulin and microtubules in the direction of microtubule assembly. The cells become blocked during the G2 and M cell cycle phases and cannot form a normal mitotic spindle and divide. Essentially, these microtubules are excessively stable and therefore dysfunctional (1).

Paclitaxel, first marketed drug of taxanes affecting the integrity of microtubules was also shown to induce Bcl-2 phosphorylation in various cancer cell lines (2). Bcl-2 is a key member of a family of proteins that control apoptosis by preventing cancer cell death and that have been involved in resistance to chemotherapy and radiotherapy. Estradiol (E2) treatment significantly increased Bcl-2 protein expression and tamoxifen blocked Bcl-2 expression (3-5). The ability of E2 to act as a survival factor for breast cancer is not well

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understood, but a substantial part of the effects is estimated to occur through the prevention of programmed cell death (apoptosis).

Estrogen receptor (ER) and its ligand, estradiol, play a significant role in the development, progression, treatment, and outcome of breast cancer. Estrogen-induced proliferation of mammary and uterine epithelial cells is primarily mediated by ER. The ER CpG islands are unmethylated in normal breast tissue and most ER-positive tumor cell lines, whereas it is methylated in about 50% of unselected primary breast cancers and most ER-negative breast cancer cell lines (6,7). The methylation of these CpG cluster sites is associated with either reduced or absent ER expression. Hypermethylation of CpG islands in the 5'-region of the ERα gene has been found in different tumor types, including breast cancer and its expression has been demonstrated to reactivate after treatment with 5-aza-2'-deoxycytidine (8). One of the promising predictive factors for paclitaxel treatment was reported to be the expression of ER. In this study, we investigated the relationship between chemosensitivity to taxanes and expression of ERα in breast cancer cell lines.

Materials and methods

Cell lines, reagents and treatments. KPL-1 and KPL-4 cells were kindly provided by Dr Junichi Kurebayashi (Kawasaki Medical School, Okayama, Japan) (9). MDA-MB-231 cell line was obtained from ATCC. S30 and JM6 cells were kindly provided by Dr V. Craig Jordan (Fox Chase Cancer Center, USA). ZR-75-1, ZR-75-30, MCF7 and T47D cells were obtained from Dainippon Sumitomo Pharma, Tokyo, Japan. ZR-75-1, ZR-75-30 and T47D cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), HEPES, streptomycin and penicillin. KPL-1 and KPL-4 cell lines were cultured in DMEM supplemented with 10% FBS, streptomycin and penicillin. MCF7 cell line was cultured in MEM containing 10% FBS, non-essential amino acid (NEAA), 1 mM sodium pyruvate, streptomycin and penicillin. MDA-MB-231 cell line was cultured in Levovitz supplemented with 10% FBS, streptomycin and penicillin. S30, JM6 were cultured in MEM (phenol red-free) supplemented with 10% Charcoal/Dextran treated FBS (Hyclone), L-glutamine, NEAA, antibiotic-antimycotic solution, insulin and G418. 5-aza-deoxycytidine, MTT and 17ß-estradiol were obtained from Sigma.

Immunostaining. Estrogen receptor expressed in human breast cancer cell lines was detected using monoclonal mouse anti-human estrogen receptor, clone 1D5 (Dako). Cells were seeded on 2-well chamber slides (Nalge Nunc) and cultured for 48 h. Cells were fixed in 4% paraformaldehyde at 4°C for 20 min, washed in PBS. Endogenous peroxidase was blocked by incubating slides with 3% H_2O_2 in methanol for 10 min, and slides were washed in PBS. After blocking of non-specific staining for 10 min (Protein Block Serum-Free, Dako), slides were incubated 1 h at room temperature with anti-ERα antibody and washed in PBS. Slides were then incubated with secondary antibody (Envision+System-HRP Labeled Polymer Anti-mouse, Dako) and washed in PBS. Immunostaining was developed using DAB Substrate-Chromogen (Liquid DAB+Substrate Chromogen System, Dako) and counterstaining was performed with Mayer's haematoxylin. Slides were dehydrated in a series of alcohol and xylene.

MTT assay for cell viability. Human breast cancer cell lines seeded in 96-well plates (5,000 cells/well) in 0.1 ml medium. Cells were cultured for 48 h before the addition of the drug. Paclitaxel, tamoxifen and 17ß-estradiol (E2) was diluted into 10% FBS-containing medium and added to each well in a volume of 0.1 ml. Cells were incubated at 37°C for 24, 72 and 120 h. MTT (20 μl) (5 mg/ml in PBS) was added to each well and incubated for ~3-4 h at 37°C. The medium was removed, the blue formazan dye was dissolved in 100 μl 0.04 N HCl isopropanol and absorption was calculated at dual wavelength (540/620 nm) using microplate reader.

Apoptosis assay. For quantitation of apoptosis induced by paclitaxel, we used CPP32/Caspase-3 Fluorometric Protease Assay Kit (MBL). Caspase-3 activity assay was performed according to the protocol recommended by the manufacturer. Briefly, cells were seeded onto 6-well plates at 1×10^5 cells/well and precultured at 37°C for 48 h. Then cells were treated with paclitaxel for 36 h, washed once with PBS, and collected by careful scraping. Treated cells were suspended in cell lysis buffer at 4°C for 40 min and protein concentrations were determined using the Bio-Rad protein determination method (Bio-Rad Laboratories, Hercules, CA). Protein (25 μg) in 50 μl of cell lysis buffer was added with 50 μl of reaction buffer and 5 μl of the 1 mM DEVD-AFC substrate and incubated at 37°C for 1 h. Samples were read in a fluorometer (Spectrofluorophotometer RF-1500; Shimadzu, Kyoto, Japan) with a 390 nm excitation filter and a 510-nm emission filter.

5-aza-2'-deoxycytidine treatment. Cells were seeded at 5×10^5 cell/100-mm culture dish. After 48 h of incubation, cells were treated with 5-aza-2'-deoxycytidine at concentrations ranging from 50 ng/ml to 5 μg/ml for 96 h. Cells treated with 5-aza-2'-deoxycytidine were exposed to RT-PCR, MTT assay and immunostaining.

RT-PCR. RNA was extracted from the cells treated with E2 by using the RNAZol. RT-PCR was performed using Perkin-Elmer GeneAmp RT PCR Kit. PCR products were analyzed by agarose (1%) gel electrophoresis and visualized by ethidium bromide staining, and the sizes were estimated by comparison to DNA molecular weight marker. Oligonucleotide sequences of PCR primers for Bcl-2, Bax were previously reported (4). Primers for Bid was designed in our laboratory: Bcl-2, sense 5'-GGT GCC ACC TGT GGT CCA CCT G-3'; antisense 5'-CTT CAC TTG TGG CCC AGA TAG G-3'. Bax, sense 5'-ATG GAC GGG TCC GGG GAGCAG C-3'; antisense 5'-CCC CAG TTG AAG TTG CCG TCA G-3'. Bid, sense 5'-GCA GGC CTA CCC TAG AGA CA-3'; antisense 5'-GTC CAT CCC ATTT TCT GGC TA-3'.

Western blotting. Total cell protein was extracted from ~20,000 cells with E2 for up to 0, 12, 24 and 48 h. Total protein (40 μg), as determined by Bio-Rad assay, was subjected to 12% SDS-PAGE and transferred to membrane (Amersham

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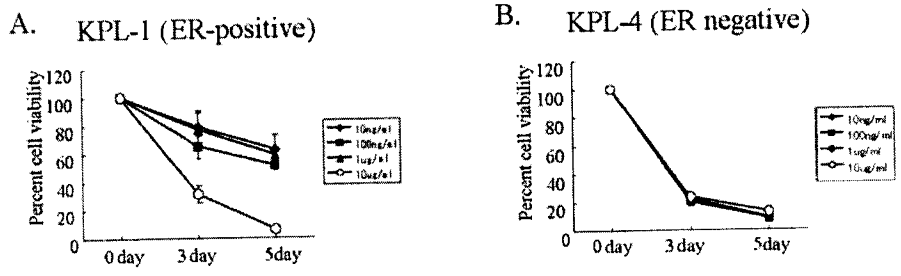

C. IC50 Values

ER positive		ER negative	
ZR-75-1	>1000 ng/ml	ZR-75-30	413 ng/ml
KPL-1	>1000 ng/ml	MDA-MB-231	37 ng/ml
T47D	>1000 ng/ml	KPL-4	0.01 ng/ml
MCF7	518 ng/ml		

Figure 1. Cytotoxic effects of paclitaxel in (A) ER-positive and (B) ER-negative cell lines by using MTT assay. (C) All of the ER-positive cells were resistant to paclitaxel, whereas ER-negative cells were sensitive to the drug.

Life Science Hybond-P). The membrane was blocked with 5% skim milk in 0.2% Tween-20 in TBS (TBST) for 1 h at room temperature and then incubated with primary antibody (Bcl-2, Bax, Santa Cruz Biotechnology Inc., Bid, BD) in TBST at 4°C overnight. The membrane was washed three times with TBST and incubated with a peroxidase-linked secondary antibody at room temparature for 1 h by shaker incubator and then washed in TBST three times. ECL Western Blotting Detection System (GE Healthcare) was used to detect secondary probes.

PowerBlot Western array analysis. Protein was extracted according to the Becton-Dickinson protocol, and 400 μg of protein were loaded in one big well across the width of the SDS-polyacrylamide gel. The gel was run overnight, and was transferred to an Immobilon-P membrane (Millipore). After transfer, the membrane was blocked for 1 h with 5% milk and clamped with a Western blotting manifold that isolates 45 channels across the membrane. In each channel, a complex antibody mixture was added and allowed to hybridize for 1 h. The blot was removed from the manifold, washed and hybridized for 30 min with secondary antibody. The membrane was developed with chemiluminescence (SuperSignal West Pico, Pierce). Data analysis includes raw and normalized digital data from each blot with changes >1.25-fold indicated. The expression data are reported as a confidence level based on fold change, reproducibilty and signal intensity.

Results

Paclitaxel-induced cytotoxicity. First we compared the cytotoxic effects of paclitaxel in 4 ER-positive and 3 ER-negative cell lines by using MTT assay. All of the ER-positive cells were resistant to paclitaxel, whereas ER-negative cells were sensitive to the drug. Each of the ER-positive cells showed IC_{50} level of >500 ng/ml, while it was 413 ng/ml, 37 ng/ml and 0.01 ng/ml for the each of ER-negative ZR-75-30, MDA-MB-231 and KPL-4 cells, respectively (Fig. 1). This result suggests that ER expression may influence chemosensitivity to paclitaxel.

Paclitaxel-induced apoptosis. To quantify the induction of apoptosis, KPL-1 (ER-positive) and KPL-4 (ER-negative) cells were treated with paclitaxel (with a range from 1 ng/ml to 1000 ng/ml) for 36 h and caspase-3 assay was performed. A dose-dependent increase in the activity of caspase-3 in KPL-4 cells was detected, while no such effect was shown in KPL-1 cells (Fig. 2). No caspase-3 activity was detected in the dose of 1,000 ng/ml in ER-negative KPL-4 cells possibly due to extensive cell death. These data suggest that at least a part of paclitaxel cytotoxicity occurs through apoptotic pathway.

Induction of ER expression and its effect to the paclitaxel chemosensitivity by 5-aza-2'-deoxycytidine in breast cancer cell lines. Methylation of ER promoter is one of the major mechanisms for the loss of ER expression in breast cancer cells. To restore ER expression, we treated two ER-negative cell lines, KPL-4 and ZR-75-30, with 5-aza-2'-deoxycytidine for 96 h and MTT assay for cell proliferation was performed. Immunostaining of KPL-4 cells demonstrated the restoration of ER expression after treatment with 5-aza-2'-deoxycytidine (Fig. 3a). The paclitaxel-sensitive KPL-4 cells became resistant in a dose-dependent manner when treated with 5-aza-2'-deoxycytidine, suggesting that reactivation of ER expression results in the gain of resistance in the cells (Fig. 3b). Similar data were obtained in ZR-75-30 cells (data not shown).

Protein expression of proapoptotic and antiapoptotic molecules after paclitaxel treatment in ER-positive and negative cells. To investigate the differences in the protein expression of proapoptotic and antiapoptotic genes, we performed Western blotting in the ER-positive (ZR-75-1, T47D, MCF7 and KPL-1) and -negative cells (ZR-75-30,

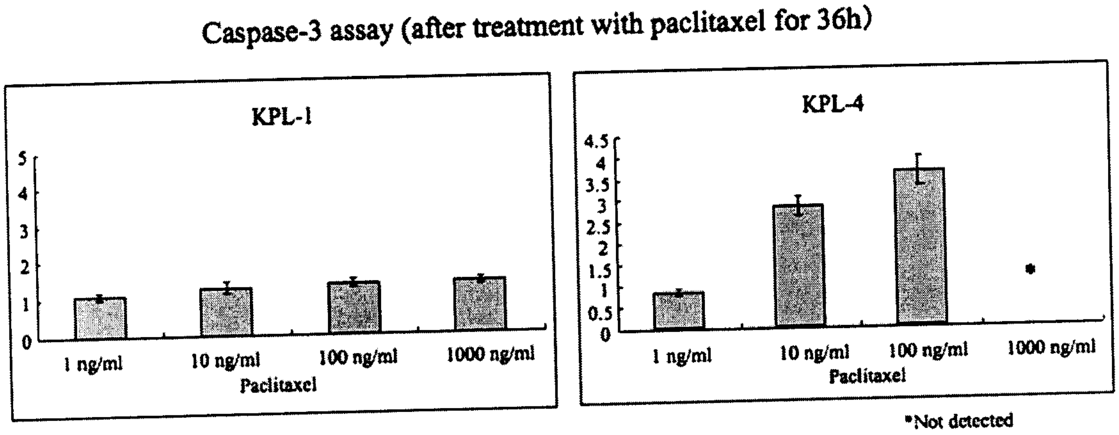

Figure 2. Caspase-3 assay after treatment with paclitaxel. A dose-dependent increase in the activity of caspase-3 in KPL-4 cells was detected, while no such effect was shown in KPL-1 cells.

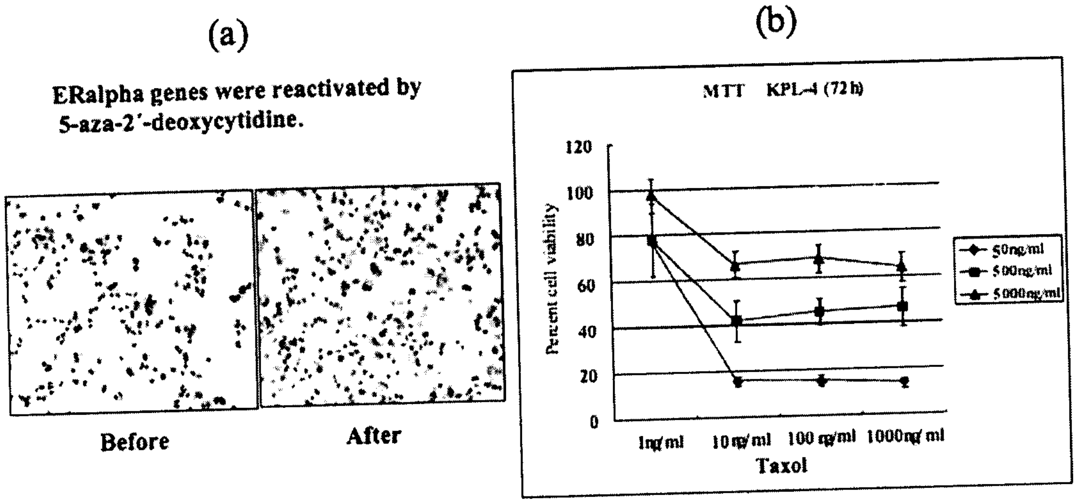

Figure 3. Induction of ER expression and its effect to the paclitaxel chemosensitivity by 5-aza-2'-deoxycytidine in breast cancer cell lines (a) and cell proliferation by MTT assay (b). Immunostaining of KPL-4 cells demonstrated the restoration of ER expression after treatment with 5-aza-2'-deoxycytidine (a). The paclitaxel-sensitive KPL-4 cells became resistant in a dose-dependent manner when treated with 5-aza-2'-deoxycytidine (b).

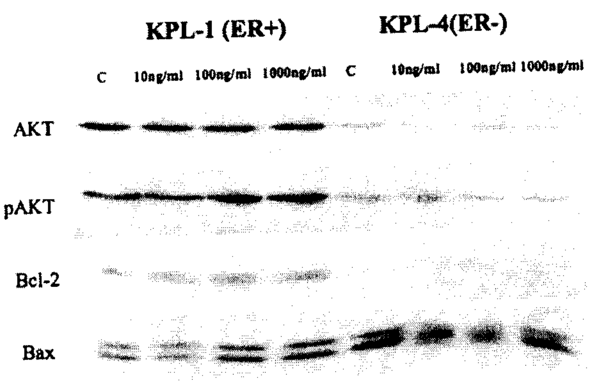

Figure 4. Protein expression of proapoptotic and antiapoptotic genes in ER-positive and -negative cells. Although proapoptotic molecule Bax expression was detected both in ER-positive and -negative cells, the expression of antiapoptotic molecules Bcl-2, Akt and phospho-Akt (pAkt) were revealed at much higher level in ER(+) KPL-1 cells as compared to ER(-) KPL-4 cells.

KPL-4) treated with various doses of paclitaxel. Although proapoptotic molecule Bax expression was detected both in ER-positive and -negative cells, the expression of anti-apoptotic molecules Bcl-2, Akt and phospho-Akt (pAkt) were revealed at much higher level in ER(+) KPL-1 cells as compared to ER(-) KPL-4 cells (Fig. 4). This pattern of the gene expression in the cells treated with paclitaxel suggested that the Bcl-2 might have a role in the resistance of ER(+) cells to paclitaxel.

Effects of estradiol (E2) on expression of Bcl-2, Bax and Bid mRNA. To determine the effects of estradiol (E2) on the expression of Bcl-2, Bax and Bid mRNA in ER(+) (ZR-75-1, T47D, MCF7 and KPL-1) and ER(-) (KPL-4, ZR-75-30) cell lines, we performed RT-PCR in the conditions induced by E2. E2 (100 nM) induced the Bcl-2 mRNA levels in ER-positive cell lines in a time-dependent manner, while there was no detectable expression of Bcl-2 in ER(-) KPL-4 cells. On the other hand, there was no significant difference

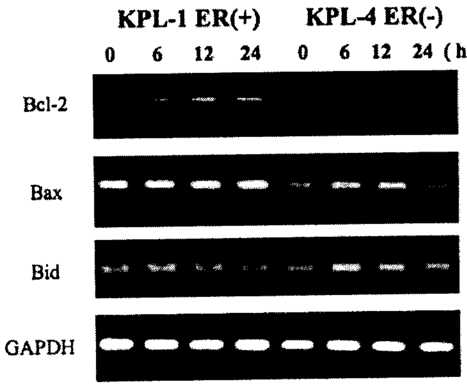

Figure 5. Effects of estradiol (E2) on the expression of Bcl-2, Bax and Bid mRNA in ER(+) and ER(-) cell lines. E2 induced Bcl-2 expression in ER-positive cell lines in a time-dependent manner, while there was no detectable Bcl-2 in ER(-) KPL-4 cells. On the other hand, no significant difference was detected for Bax and Bid mRNA levels before or after E2 treatment either in ER(+) or ER(-) cells.

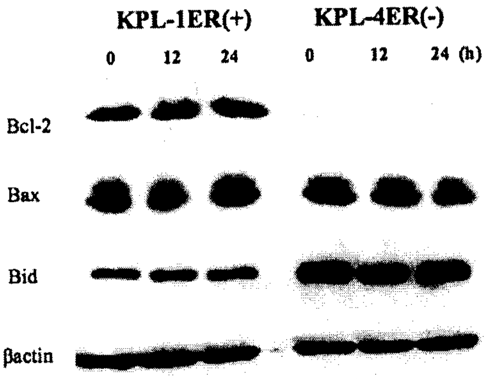

Figure 6. Effects of estradiol (E2) on the expression of Bcl-2, Bax and Bid proteins in the ER-positive and -negative cell lines. An increase in Bcl-2 protein was detected in ER(+) KPL-1 cells, whereas no Bcl-2 expression was detected in ER(-) KPL-4 cells. On the other hand, no significant difference in Bax and Bid protein expression was detected either in ER(+) KPL-1 or and ER(-) KPL-4 cells.

in Bax and Bid mRNA levels before or after E2 treatment either in ER(+) or ER(-) cells (Fig. 5).

Effects of estradiol (E2) on expression of Bcl-2, Bax and Bid proteins. To determine the effects of estradiol (E2) (100 nM) on the expression of Bcl-2, Bax and Bid protein in the ER-positive and -negative cell lines, Western blotting was performed. 0, 12 and 24 h after treatment with E2, an increase in Bcl-2 protein was detected in ER(+) KPL-1 cells, whereas no Bcl-2 expression was detected in ER(-) KPL-4 cells similar to the mRNA expression (Fig. 6). On the other hand, no significant difference in Bax or Bid protein expression was detected either in ER(+) KPL-1 or ER(-) KPL-4 cells similar to mRNA expression pattern.

Expression of Bcl-2 after treatment with 5-aza-2'-deoxycytidine in ER(-) KPL-4 cells. When ER(-) KPL-4 cells were treated with 5-aza-2'-deoxycytidine for 96 h and then induced by estradiol, a restoration of Bcl-2 expression was detected (Fig. 7). These data suggested a link between ER and anti-apoptotic molecule Bcl-2 expression, which may explain the resistance to paclitaxel in ER(+) cells.

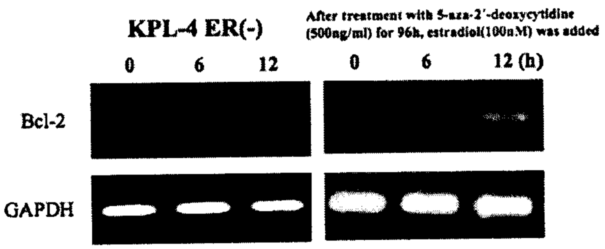
KPL-4 ER(-)
After treatment with 5-aza-2'-deoxycytidine (500ng/ml) for 96h, estradiol(100nM) was added
0 6 12 0 6 12 (h)
Bcl-2
GAPDH

Figure 7. Expression of Bcl-2 after treatment with 5-aza-2'-deoxycytidine in ER(-) KPL-4 cells. When ER(-) KPL-4 cells were treated with 5-aza-2'-deoxycytidine for 96 h and then induced by estradiol, a restoration of Bcl-2 expression was detected.

ER expression in parent MDA-MB-231 and its ER-stably transfected cell lines. We examined ER expression in MDA-MB-231 and its wild-type and mutant ER stably transfected cells (S30 and JM6, respectively). Immunohistochemistry showed no expression of ER in MDA-MB231 cells, while its stable transfectants, S30 and JM6, demonstrated ER protein (Fig. 8a). The cell lines were furthermore subjected to paclitaxel treatment at various doses and the chemosensitivity was measured. While the parent ER(-) MDA-MB-231 cells were sensitive to paclitaxel treatment, its ER stable transfectant S30 and JM6 cells revealed resistance to the drug at all doses (Fig. 8b).

To identify the protein expression patterns of MDA-MB-231 and its stable transfectants, PowerBlot microarray system was used. This system can detect the relative levels of 40 different proteins in two groups of cell lines. The Power Blot analysis revealed a clear-cut difference in the expression of some proteins. The proapoptotic protein Bid was decreased in ER stable transfectants from 4- to 28-fold as compared to the ER(-) parent cells, whereas Cox-2 and NF-κB p65 proteins were enhanced from 2 to 10 times as compared to the control parent cells (data not shown). Increase or decrease in the protein expression of some other molecules such as PI3-kinase (4-fold decrease) and EGFR (3-fold increase) in JM6 cells as compared to MDA-MB-231 cells, suggesting that some signaling pathways in wild-type and mutant ER stable cells might be different (data not shown).

In light of the data obtained from PowerBlot analysis, we examined the Bid protein expression by Western blotting. Western blot analysis showed a decrease of Bid in S30 cells and almost total loss in JM6 cells, suggesting that the paclitaxel sensitivity in MDA-MB-231 cells, but resistance in its ER-stable cells, could be due to regulation of Bcl-2 family proteins, anti-apoptotic molecules, Bcl-2 as well as Bcl-x, and pro-apoptotic molecules, Bax and Bid (Fig. 9).

Discussion

ER is a member of the nuclear receptor superfamily of transcription factors. ER activation of gene expression is dependent on ligand, cellular, and gene promoter context and

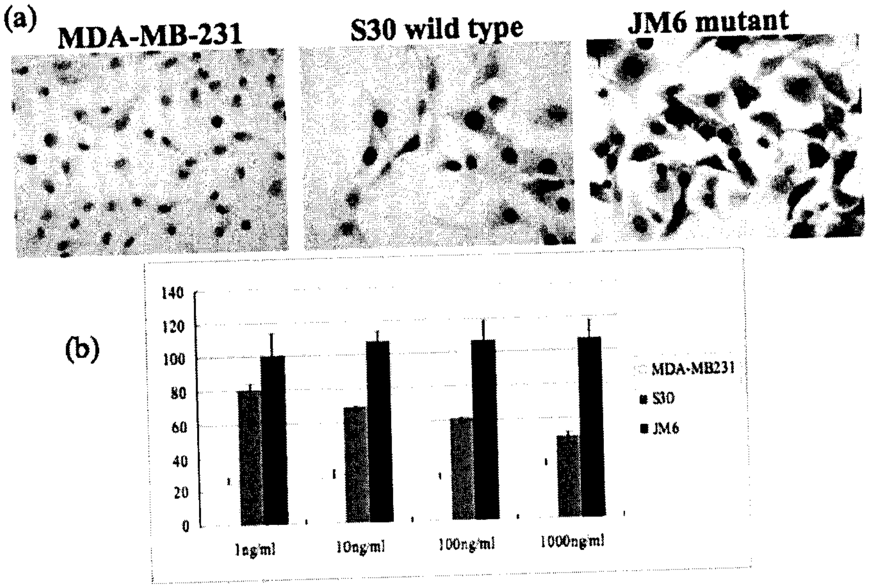

Figure 8. ER expression in parent MDA-MB-231 and its ER-stably transfected S30 and JM6 cell lines. Immunohistochemistry showed no expression of ER in MDA-MB231 cells, while its stable transfectants, S30 and JM6, demonstrated ER protein (a). MTT assay after paclitaxel treatment in various doses showed chemosensitivity of the parent ER(-) MDA-MB-231 cells but resistance of its ER stable transfectants S30 and JM6 cells in all doses (b).

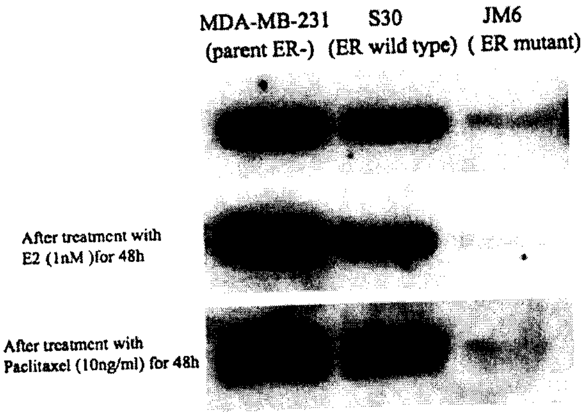
MDA-MB-231 S30 JM6
(parent ER-) (ER wild type) (ER mutant)
After treatment with E2 (1nM)for 48h
After treatment with Paclitaxel (10ng/ml) for 48h

Figure 9. Bid protein expression by Western blotting based on the data obtained from PowerBlot analysis. The analysis showed a decrease of Bid in S30 cells and almost total loss in JM6 cells, suggesting that paclitaxel sensitivity in MDA-MB-231 cells but resistance in its stable transfectants.

receptor isoform. ER is a critical growth-regulatory gene in breast cancer, and its expression status is tightly linked to the prognosis and treatment outcome of breast cancer patients.

Paclitaxel is a drug used in treatment of cancer. The cytotoxicity of paclitaxel is linked to both inhibition of cell proliferation and apoptosis. Our results indicated that ER-negative breast cancer cell lines were more sensitive to paclitaxel than ER-positive breast cancer cell lines. The molecular consequences of paclitaxel exposure and the resultant induction of DNA fragmentation and apoptotic cell death have been documented in breast and ovarian cancers (1,10,11). We have demonstrated that paclitaxel, a chemotherapeutic agent that is widely used in the clinical treatment of breast cancer, induced apoptosis in KPL-4 breast cancer cells after 36 h of treatment and detected dose-dependent increased activity of caspase-3. Paclitaxel has been shown to trigger cell death primarily via caspase-independent routes in the non-small cell lung cancer cell line (12). Okano and Rustgi (13) recently reported that physiologically relevant concentrations of paclitaxel caused cell death via both caspase-dependent and -independent pathways in human esophageal squamous cancer cells.

The expression of anti-apoptotic molecules Bcl-2, Akt and phospho-Akt (pAkt) were revealed at much higher level and the expression of proapoptotic molecule Bid was revealed at lower level in ER(+) KPL-1 cells as compared to ER(-) KPL-4 cells. A direct interaction between tubulin and several pro-apoptotic and anti-apoptotic members of the Bcl-2 family has been demonstrated by effects on the assembly of microtubules from pure rat brain tubulin (14).

Estrogens stimulate growth of hormone-responsive breast cancer cells through complex and still incompletely characterized mechanisms. We have demonstrated that estradiol increased Bcl-2 mRNA level and proteins in ER-positive cell lines. However, no significant changes in Bax mRNA level and proteins in breast cancer cell lines. These results are in agreement with a previous report where estrogen up-regulated the anti-apoptotic Bcl-2 gene, whereas Bax level was not affected by E2 in breast cancer cell line (4,5,15-17).

In breast cancer cell lines or tumor specimens, altered expression of host genes has been associated with aberrant DNA methylation, including BRCA1, RARß, 14-3-3, Wilms tumor 1, p16, uPA, estrogen receptor, E-cadherin, maspin, and others (8,18-20). DNAs from human ER-negative breast cancer cell lines have been shown to be highly methylated at the CpG Island of ER gene as determined by Southern blot analysis (21). Upon treatment with the demethylating agent 5-aza-2'-deoxycytidine, the ER gene CpG Island was demethylated resulting in re-expression of a functional ER (22). We showed that 5-aza-2'-deoxycytidine induced ER gene expression in ER-negative breast cancer cell lines, KPL-4 and ZR-75-30.

After ER gene expression in KPL-4 was activated by 5-aza-2'-deoxycytidine, E2 induced time-dependent up-regulation of Bcl-2 mRNA. In breast carcinomas, Bcl-2 has been shown to associate with ER status (23). Thus, Bcl-2 may be regulated through the interaction of estrogen with ER.

In conclusion, our results demonstrate that ER expression may influence chemosensitivity to paclitaxel through anti-apoptotic gene regulation. Regulation of the pathway may be crucial to increase the efficacy of taxanes in ER-positive breast cancer.

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Early breast cancer

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Adoption of urbanised lifestyles together with changes in reproductive behaviour might partly underlie the continued rise in worldwide incidence of breast cancer. Widespread mammographic screening and effective systemic therapies have led to a stage shift at presentation and mortality reductions in the past two decades. Loco-regional control of the disease seems to affect long-term survival, and attention to surgical margins together with improved radiotherapy techniques could further contribute to mortality gains. Developments in oncoplastic surgery and partial-breast reconstruction have improved cosmetic outcomes after breast-conservation surgery. Optimum approaches for delivering chest-wall radiotherapy in the context of immediate breast reconstruction present special challenges. Accurate methods for intraoperative assessment of sentinel lymph nodes remain a clinical priority. Clinical trials are investigating combinatorial therapies that use novel agents targeting growth factor receptors, signal transduction pathways, and tumour angiogenesis. Gene-expression profiling offers the potential to provide accurate prognostic and predictive information, with selection of best possible therapy for individuals and avoidance of overtreatment and undertreatment of patients with conventional chemotherapy. Short-term presurgical studies in the neoadjuvant setting allow monitoring of proliferative indices, and changes in gene-expression patterns can be predictive of response to therapies and long-term outcome.

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Introduction

Breast cancer remains the most common malignancy in women worldwide and is the leading cause of cancer-related mortality.[1] More than 1·2 million cases are diagnosed every year, affecting 10–12% of the female population and accounting for 500 000 deaths per year worldwide. Despite a higher prevalence of breast cancer in industrialised than in non-industrialised countries, incidence rates are steadily increasing in less affluent societies.[2] Breast cancer is mainly a postmenopausal disease, with more than three-quarters of tumours hormone responsive. This hormone dependency interacts with environmental and genetic factors to determine incidence and progression of the disease. Lifestyle and environmental effects are potentially modifiable risk factors and offer the prospect of interventions that might ultimately reduce the global burden of the disease.[3] The documented decrease in breast-cancer rates in the USA in 2003, after a gradual rise during the preceding 30 years, exemplifies the effect of risk modification. This reduction has been linked to decreased use of exogenous hormones after adverse reports of an association with increased breast-cancer risk[4] and a possible reduction in the uptake of screening mammography in view of its debatable modest benefit.

Mortality rates for breast cancer have fallen in many industrialised nations since around 1990, having previously been stable or increasing for several consecutive decades.[5–7] These falls in mortality have been attributed mainly to the introduction of mammographic screening programmes and the widespread use of adjuvant systemic therapies with tamoxifen.[8] A US population-based study showed that these mortality trends are accentuated in women with oestrogen-receptor-positive tumours compared with those with hormone-insensitive disease.[9] Moreover, this decrease in mortality was almost exclusively confined to women younger than 70 years (figure 1). This Seminar will focus on the epidemiology, diagnosis, and management of early breast cancer when there is no overt evidence of distant metastases and for which treatment intent is curative. Some patients can be cured with loco-regional treatment alone whereas many will have undetectable micrometastatic disease and require adjuvant systemic therapy.

Epidemiology

Age and female sex are major risk factors for breast cancer, with incidence rates rising rapidly between the ages of 35 and 39 years and subsequently levelling to a plateau after 80 years.[10] Nonetheless, the rate of increase slows around the age of 50 years, corresponding to the average age of menopause, which creates a point of inflection in the age-specific incidence curve known as Clemmesen's hook (figure 2).[11] This transition point is an indicator of the confluence of two separate rate curves for oestrogen-receptor-positive and negative tumours that have fairly favourable and poor prognoses, respectively.[10] The incidence of oestrogen-receptor-negative tumours increases rapidly until age 50 years and then flattens or decreases. By contrast, the incidence of oestrogen-receptor-positive tumours is similar up to the age of 50 years, but then continues to climb at a slower pace.

Search strategy and selection criteria

We searched the Cochrane Library and Medline between 1998, and 2008, with the term "breast cancer". In section of publications, we used discretion from our perception of the importance of the articles on the basis of citation within the medical published work and at major international conferences (San Antonio Breast Cancer Symposia; American Society of Clinical Oncology meetings). We restricted searches to the past 5 years, but some older referenced papers were collated from our personal collections. Some review articles have been cited to provide a more comprehensive list of references than is permissible in this Seminar.

Thus oestrogen-receptor-negative tumours tend to occur earlier in life and oestrogen-receptor-positive tumours are more common in older women. The peak ages of onset for these two tumour phenotypes are 50 and 70 years of age, respectively, and they seem to have different underlying causes and pathobiology. Reproductive and anthropomorphic factors have opposing effects, with nulliparity, obesity, and oral contraceptive use decreasing the risk of early-onset breast cancers while increasing the risk in older women.[12–14]

There are pronounced racial differences in the incidence and mortality of breast cancer.[15] Although age-standardised incidence rates are higher in white women than in those of African-American descent, these rates cross at about age 50 years. At younger ages, breast-cancer incidence rates are higher in African-American women, whereas rates are greater in their white counterparts at 50 years of age and older. Age-adjusted breast-cancer mortality rates were congruent between African-Americans and white Americans until the early 1980s, but thereafter a continued divergence was evident with higher mortality rates for African-American than for white people.[16] These differential mortality rates coincided with the introduction of mammographic screening in conjunction with adjuvant systemic therapy as an integral component of breast-cancer management.[16] Black women have a higher proportion of oestrogen-receptor-negative tumours than do white women and are therefore less likely to receive endocrine treatment. Furthermore, socioeconomic variation leads to inequalities in terms of health-insurance cover and educational attainment, which are likely to restrict access to new treatments and screening programmes for ethnic groups. A combination of intrinsic differences in response and health-care provision might account for the widening racial disparity in breast-cancer mortality rates within the USA.[16,17]

Familial forms of breast cancer incorporate both high and low genetic risk. When several first-degree relatives are affected, clustering is probably hereditary and attributable to high-risk susceptibility genes such as *BRCA1* and *BRCA2*.[18] These are tumour suppressor genes that display an autosomal dominant pattern of inheritance with variable penetrance. Mutations within these two genes account for around three-quarters of hereditary breast cancer cases (5–10% of all breast cancer) and confer a lifetime risk of between 80–85% by age 70 years.[19] Additionally, *BRCA1* mutations are associated with ovarian cancer risk of 20–40%. Within cells, the effects of *BRCA1* and *BRCA2* are recessive, and both copies of an allele must be lost or mutated for cancer

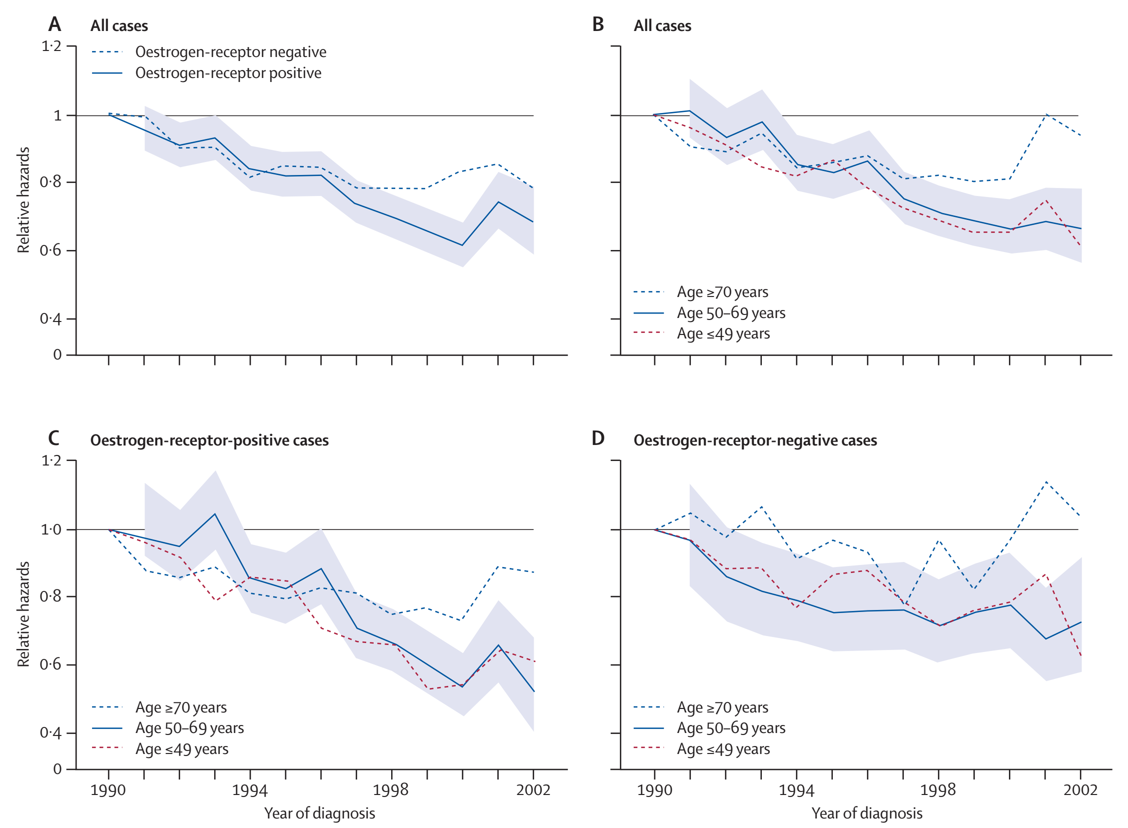

***Figure 1:* Relative hazard rates of breast-cancer death according to oestrogen-receptor status and age at diagnosis**
Shading indicates 95% CIs. Reprinted with permission from the *Journal of Clinical Oncology*.[9]

progression. Individuals with a germline mutation in these genes have a dominantly inherited susceptibility, and the second so-called hit occurs in the somatic copy. Tumours from genetically predisposed patients show loss of heterozygosity in the wild-type *BRCA1* allele,[20] but mutations of *BRCA1* and *BRCA2* are uncommon in sporadic breast cancers. Other genes involved in genetic predisposition include *p53* (Li-Fraumeni syndrome), *AT* (ataxia telangiectasia), and *PTEN* (breast and thyroid cancer). Low penetrance genes such as *CHEK* mutations might collectively be responsible for up to 25% of familial cases; although they confer a reduced risk, they are more prevalent within the population.[21]

Breast-cancer risk is modulated by factors affecting the hormonal milieu; most mammary tumours are stimulated by oestrogens, and oestrogen-receptor-negative tumours can evolve from oestrogen-receptor-positive lesions rather than arising de novo. A woman's cumulative lifetime exposure to oestrogen determines the level of this environmental risk. Thus early menarche (<12 years *vs* 16 years) and late menopause (>55 years *vs* <45 years) are associated with relative risk increases of about 1·2.[3] Levels of oestrogen and rates of proliferation in breast epithelium are low after menopause, which when induced iatrogenically at younger than 40 years of age reduces the risk of breast cancer by almost two-thirds.[3] A first full-term pregnancy at younger than 20 years of age is protective for breast cancer, and high circulating concentrations of progesterone can cause terminal differentiation in pluripotential stem cells of immature breast tissue. Nulliparity is a well known risk factor for breast cancer since Ramizzini described *horrendis mammarium canceris* in Catholic nuns.[22] However, women who defer childbearing beyond 35 years of age have an increased relative risk compared with nulliparous women, which might have relevance to contemporary reproductive practices in which late pregnancies are associated with prolonged use of the oral contraceptive pill and a greater chance of pregnancy-related breast cancer.[23]

Hormone-replacement therapy increases the relative risk of breast cancer by roughly 35% after 10 years of use, although cancers developing in women who have ever used this therapy tend to be of more favourable prognosis.[24] Hormone-replacement therapy should be avoided in breast-cancer survivors, and more than doubles the risk of recurrence.[25] Moderately vigorous physical activity of up to 7 h per week can reduce the risk of breast cancer by almost 20%, and this effect is independent of menstrual function. Daily strenuous exercises reduce risk by up to 50% in women aged 14–22 years.[26] Alcohol consumption increases breast-cancer risk irrespective of the type of beverage consumed.[27] High mammographic breast density is a powerful independent predictor of breast-cancer risk and is associated with an increased ratio of glandular to fatty tissue.[28] Only a quarter of sporadic cases of breast cancer have any identifiable risk factor.

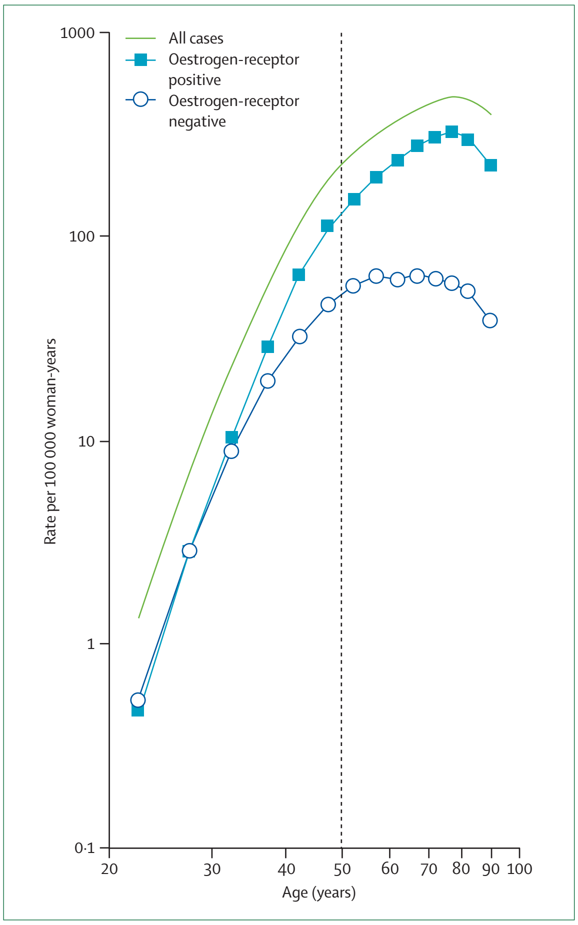

Figure 2: **Age-specific incidence rates (SEER Database) in female patients with breast cancer, showing point of inflexion (Clemmesen's Hook) at around 50 years of age**
SEER=Surveillance, Epidemiology and End Results program of the National Cancer Institute. Reproduced with permission from Springer-Verlag.

Diagnosis

About three-quarters of symptomatic breast cancers will present with a discrete breast lump. However, most patients referred to a breast clinic with a lump will have benign disease, and initial clinical examination will aim to establish whether a dominant mass or localised glandular nodularity is present. The physical characteristics of benign and malignant breast lumps overlap substantially. Thus complete clinical evaluation involves triple assessment, integrating information from clinical examination, radiological imaging, and percutaneous needle biopsy. A diagnostic mammogram can confirm a clinical suspicion of malignancy and typically shows a spiculate opacity or microcalcification. It can sometimes show the extent of malignancy (especially when associated with microcalcification) and identify occult (non-palpable) lesions in the ipsilateral or contralateral breast. Mammography does not show evidence of malignancy in 10% of patients with breast cancer.[29]

Breast ultrasound with 12–15 MHz transducers is complementary to mammography and increases diagnostic accuracy. It provides a measurement of tumour size, correlating well with pathological estimates.[30] Modern ultrasound devices incorporate a Doppler facility and are increasingly being used to image the axillary nodes and deselect patients for sentinel-lymph-node biopsy. Tissue diagnosis is essential and can be obtained with either fine-needle aspiration cytology or core biopsy. Before image-guided biopsy techniques, most tissue acquisition involved open excision or incision biopsy. Percutaneous needle-biopsy techniques can now provide a definitive diagnosis for most benign and malignant diseases.[31] Although fine-needle aspiration cytology simply, quickly, and cost-effectively establishes tissue diagnosis, it has lower sensitivity and specificity than does core biopsy.[31] Despite false-negative results with both, core biopsy with wide-bore needle is preferred to fine-needle aspiration cytology and yields solid cores of tissue that maintain tissue architecture and allow distinction between invasive and non-invasive carcinoma.[32] Biopsy samples of mass lesions can be taken with ultrasound or stereotactic guidance, whereas microcalcification usually mandates stereotactic methods.[33] The standard core-biopsy needle is either 14 or 16 gauge, but larger volumes of tissue can be obtained from vacuum-assisted core biopsy devices with a range of needle sizes, but most often an 11 gauge needle. Vacuum-assisted core-biopsy devices reduce the chance of underdiagnosis and increase the chance of obtaining a definitive preoperative diagnosis, allowing appropriate planning of breast-cancer surgery.[34]

When clinical examination, radiology, and core biopsy or fine-needle aspiration cytology show benign features only, the probability of malignancy is very low. A diagnostic (surgical) excision biopsy is warranted in the absence of concordance, although repeat core-needle biopsy can be attempted. MRI is used selectively in the diagnostic workup of breast-cancer patients to clarify the extent of a lesion and establish whether satellite foci are present in patients otherwise amenable to breast-conservation surgery.[35] However, evidence suggests that patients assessed with MRI are more likely to undergo (unnecessary) mastectomy instead of breast-conservation surgery.[36,37] Rates of ipsilateral breast tumour recurrence (IBTR) are fairly low—at 8 years rates are similar in patients receiving breast-conservation surgery with (3%) and without (4%) preoperative MRI imaging[38]—and additional lesions detected by MRI might not be clinically relevant or might be adequately treated with adjuvant therapies.[39]

A substantial proportion of breast cancers in the USA and western Europe are detected with screening mammography. Randomised controlled trials have confirmed that screening mammography with or without clinical breast examination in postmenopausal women reduces breast-cancer mortality by about 20%.[40] The use of screening mammography in premenopausal women remains controversial, and is probably not cost effective.[41] Some suggest that clinical breast examination should accompany mammographic screening, since some cancers are radiologically occult but clinically palpable.[42] Breast self-examination has not yet proven beneficial in clinical trials.[43] Breast MRI screening has been recommended for high-risk women with *BRCA1/2* mutation carriage.[44] The sensitivity of breast MRI and cancer-detection rates within this group are better than with mammography; however, data from prospective randomised controlled trials assessing the effect on breast-cancer mortality are scarce.

Biological hypotheses

Two biological notions of tumour pathogenesis have guided strategies for loco-regional and systemic treatment of breast cancer.[45] According to the Halstedian paradigm, breast cancer is a localised disease at inception with progressive and sequential spread from local tissues to lymph nodes and in turn haematogenous dissemination. IBTR is considered a cause of distant metastases, with the chance of cure related to the extent of primary loco-regional treatment. The Fisherian paradigm presupposes that breast cancer is predominantly a systemic disease at the outset, with cancer cells entering the bloodstream at an early stage of tumour development. Circulating tumour cells might be destroyed by the immune system, but some will establish viable micrometastatic foci at distant sites. Micrometastases at the time of diagnosis will determine a patient's clinical fate. IBTR is regarded as an indicator of distant-relapse risk and indicates a host-tumour relation that favours development of distant disease or activation of processes leading to a kick start of micrometastases. This notion of biological predeterminism has dominated approaches to breast-cancer management over the past three decades and emphasised the importance of systemic therapies targeting distant micrometastatic disease. Long-term follow-up of the largest breast conservation trial (NSABP B-06) at 20 years suggests that variations in extent of loco-regional treatments do not affect overall survival,[46] supporting the idea that local recurrence is an indicator of risk for development of distant disease that reflects intrinsic biology of the tumour.[47] Several studies have shown that IBTR is the strongest independent predictor of distant relapse, conferring an increased risk of up to three-fold to four-fold.[48] Although IBTR contributes roughly a third to the overall recurrence risk (Blamey R, University of Nottingham, personal communication), whether IBTR is causally related to distant relapse or merely associated with survival is unknown.

In a meta-analysis by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG), local radiation treatment to either the breast after breast-conservation surgery or the chest wall after mastectomy showed an overall survival benefit at 15 years.[49] For treatment comparisons in which the difference in local recurrence rates at 5 years was less than 10%, survival was unaffected. When differences in local relapse were substantial (>10%), moderate reductions in breast-cancer-specific and overall mortality were recorded. Absolute reductions were 19% for local recurrence at 5 years and 5% for breast-cancer mortality at 15 years, representing one life saved for every four loco-regional recurrences prevented by radiotherapy at 5 years. This analysis showed conclusively that differences in loco-regional treatments that substantially improve rates of local control will affect long-term survival of patients with breast cancer. Local control does matter and rates of local recurrence should be kept to a minimum in the first 5 years. Up to a quarter of local recurrences will be a determinant and not simply an indicator of risk for distant relapse and death.

Molecular profiling can help to predict the biological behaviour and pattern of spread for individual tumours and avoid undertreatment and overtreatment with both loco-regional and systemic therapies.[50] Malignant stem cells are either quiescent or cycle fairly slowly, and are resistant to conventional chemotherapy. Their ability to self-renew provides the opportunity for regeneration and clinical recurrence of cancer. Identification of biochemical pathways that are unique to cancer stem cells will allow selective targeting of this important subpopulation of tumour cells. Cellular response to therapies should be anticipated and escape mechanisms co-targeted.

Surgery

The introduction of conservative surgery for breast cancer coincided with reduced tumour size at presentation and a shift in the underlying biological hypothesis. Breast-conservation surgery is now an established procedure and the preferred standard of care for management of women with early-stage breast cancer. Instigation of widespread mammographic screening has contributed to a stage shift for newly diagnosed disease, with an average tumour size at presentation of less than 2 cm. At least two-thirds of patients are eligible for breast-conservation surgery, but rates of mastectomy vary both geographically and institutionally.[51] Selection of patients for this surgery is crucial, with an inverse relation between competing oncological demands for surgical radicality and cosmesis. A balance exists between the risk of IBTR and cosmetic results, with oncoplastic surgery advancing the limits of surgical resection.

Two factors emerge as principal determinants of true local recurrence within the conserved breast: margin status and the presence or absence of an extensive in-situ component. Lymphatic invasion and young age (<35 years) are primary predictors for increased risk of IBTR. Consistent associations have been recorded for larger tumour size (>2 cm) and higher histological grade but not for tumour subtype or nodal status.[52] Results of the EBCTCG overview have reinforced the link between local control and mortality, leading to an emphasis on adequacy of surgical excision and other treatment-related variables such as radiotherapy.[49] Attainment of gross macroscopic clearance of the tumour at operation is no longer acceptable; all radial margins should be clear of tumour microscopically. A positive resection margin has not been uniformly defined, which has compounded issues relating to microscopically negative margins and degrees of surgical clearance—eg, how wide must a negative margin be to result in acceptable rates of local recurrence (1–2% per year)? Many surgeons regard a margin clearance of 2–3 mm to be appropriate, although up to 45% of American radiation oncologists consider a margin as negative provided that no tumour cells are at the inked edge.[53] Others strive for a radial margin clearance of 5 mm, which can lead to re-excision rates of up to 50% but is associated with very low rates of IBTR. Detection of further tumour is unusual when re-excision is done to achieve a wider margin rather than a negative margin. Singletary[54] has provided a useful analysis, showing median rates of IBTR of 3%, 6%, and 2% when margins of clearance were 1 mm, 2 mm, or just clear, respectively. Thus although rates of recurrence are determined by negative margin status, no direct relation exists between margin width and rates of local recurrence. When the first re-excision fails to achieve surgical clearance, mastectomy is often indicated and becomes necessary if margins remain positive after a reasonable number of surgical attempts (up to three).[55]

Practices are consistent with the notion that IBTR develops from re-growth of residual cancer cells in peritumoral tissue. Moreover, the invasive element confers the increased risk of distant failure; when local recurrence is exclusively ductal carcinoma in situ, features of the original primary tumour will determine systemic risk.

Most patients considered suitable for breast-conservation surgery will have a unifocal tumour measuring 3 cm or less and lying more than 2 cm from the nipple areolar complex. These patients usually have a favourable ratio of tumour to breast size and are amenable to conventional forms of wide local excision in which the tumour is excised with a roughly 2 cm margin of surrounding breast tissue without the need for any formal breast remodelling. Although a re-excision might be needed in up to a quarter of cases to achieve microscopically clear radial margins, an optimum cosmetic result should be attained after irradiation of the remaining breast tissue. Notwithstanding attempts at breast-conservation surgery, mastectomy is clearly indicated for some patients on the basis of tumour size or location, multifocality, an inflammatory component, or patient choice. Achievement of a good cosmetic outcome becomes progressively more difficult as the proportion of breast tissue removed increases. Although the absolute volume of tissue excised is surgeon dependent, a greater percentage is associated with larger tumours. When more than 10–20% of breast volume is removed, results might be unsatisfactory cosmetically.[56] Partial-breast reconstruction with oncoplastic procedures often allow wide resection of tissue, increasing the chance of tumour-free margins. Despite these techniques

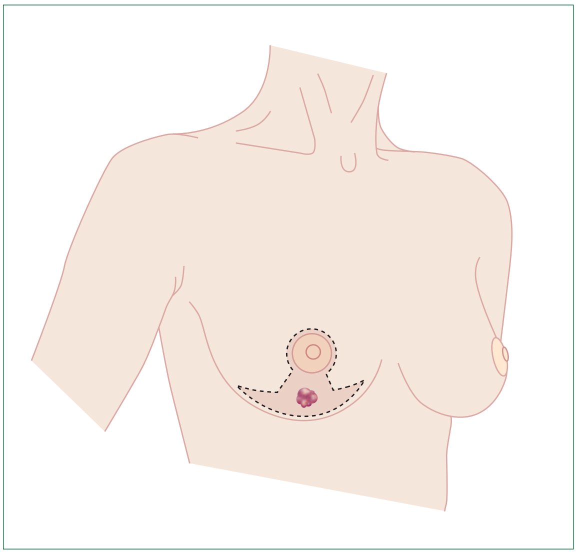

Figure 3: **A tumour in the inferior quadrant of the breast can be excised as part of a reduction mammoplasty specimen with wide surgical margins**

providing an opportunity for enhancing quality of life by improving cosmetic outcome and psychological well-being,[57] there are no long-term follow-up data to substantiate the claim for reduced rates of local relapse. Moreover, transposition of glandular tissue could jeopardise accurate targeting of any radiotherapy boost, unless it is given intraoperatively. Therapeutic mammoplasty can potentially improve cosmetic outcome when tumour size or location would otherwise lead to sub-optimum cosmesis after conventional breast-conservation surgery (figure 3).[58] Strict oncological selection criteria should still be applied;[59] when the estimated risk for IBTR is high, despite clear margins and a good cosmetic result, mastectomy with immediate breast reconstruction should be offered. The development of oncoplastic surgery and partial-breast reconstruction has improved the application of breast-conservation surgery to management of breast cancer, but careful patient selection is crucial. Often a contralateral procedure is required for symmetrisation, particularly when volume displacement rather than replacement techniques are used.[60]

Most patients who are healthy and younger than 70 years with a non-inflammatory or locally advanced tumour should be offered immediate breast reconstruction together with a skin-sparing mastectomy in which the nipple areolar complex is removed but much of the breast skin envelope remains. Skin-sparing mastectomy represents the latest phase in development of less mutilating forms of mastectomy and has revolutionised results of immediate breast reconstruction by preserving the inframammary fold and avoiding the need for resculpturing of any imported skin or tissue expansion of residual chest-wall skin. No evidence suggests increased rates of local recurrence with skin-sparing mastectomy, and the precise skin incision should be tailored to the individual patient with removal of any involved skin overlying a tumour.[61] Breast volume can be reconstituted with a variety of techniques including subpectoral tissue expander, extended autologous latissimus-dorsi flap, implant-assisted latissimus-dorsi flap, or a free/pedicled transverse rectus abdominus flap. Judicious patient selection and joint decision-making will help keep any disparity between patient expectation and clinical reality to a minimum and maximise satisfaction.

Many women now receive postmastectomy radiotherapy.[49,62,63] Anticipation of chest-wall irradiation will affect the choice of reconstructive technique; an implant-only reconstruction is generally avoided when postmastectomy radiotherapy is a possibility.[64] The potential problems of capsular contracture in this group of patients with implant-based reconstruction have led to a modified surgical approach with a delayed immediate reconstruction. A skin-sparing mastectomy can be undertaken initially with placement of a temporary tissue expander that acts as scaffolding for the skin flaps. Chest-wall irradiation can then be given and definitive reconstruction undertaken later. The viability of the native mastectomy flaps after radiation causes concern, and it might be preferable to proceed with immediate reconstruction with a latissimus-dorsi flap and implant for all patients and undertake implant exchange if and when required. An extended autologous latissimus-dorsi flap is not necessarily more tolerant of radiotherapy, and substantial donor-site morbidity occurs. However, delayed immediate reconstruction is a method that can potentially preserve the aesthetic benefits of immediate breast reconstruction with preservation of the three-dimensional skin envelope and more accurate targeting of tangential radiotherapy beams.

Methods for accurately staging the axilla continue to evolve, but remain dominated by sentinel-lymph-node biopsy, which is now widely practised and accepted as a standard of care. Dual labelling techniques with blue dye and isotope are associated with a shorter learning curve and optimum performance indicators such as rates of identification (>90%) and false negativity (5–10%).[65] Blue dye-assisted node sampling removes three to four blue and palpably suspicious nodes and can be a pragmatic and cost-effective method when radioisotope facilities are unavailable.[66] However, this method is associated with a higher false negative rate and lacks the reassurance provided by the absence of any residual radioactivity within the axilla. Results from the largest sentinel lymph node biopsy trial show an overall false negative rate of 9·8%, with higher rates when only one sentinel node is removed rather than two to three nodes.[67] Completion axillary-lymph-node dissection is recommended for all patients with either micrometastatic or macrometastatic deposits in the sentinel lymph node.[65] The chance of non-sentinel lymph-node involvement is related to the volume of disease in the sentinel node, but nomograms devised for estimation of this involvement are difficult to reliably apply in practice and are less accurate when the predicted incidence of non-sentinel lymph-node positivity is low.[68] For some patients, the risk to benefit ratio for detection of positive cases of non-sentinel lymph nodes might not justify any delayed procedure. Low rates of axillary relapse are unlikely to translate into any meaningful reduction in long-term survival in an older group of patients with smaller non-high-grade tumours.[69] Methods for intraoperative assessment of sentinel lymph nodes obviate the need for a delayed axillary-lymph-node dissection, but detection of micrometastases with either touch imprint cytology or frozen section is problematic. New techniques based on reverse-transcriptase PCR can potentially overcome difficulties of limited node sampling and operating parameters set at a threshold for detection of metastases greater than 0·2 mm in size but not isolated tumour cells (≤0·2 mm).[70] Real-time PCR might allow quantitation and differentiation between macrometastases and micrometastases.

Rates of clinical regional recurrence in patients with negative sentinel-lymph-node biopsy who have not proceeded to axillary-lymph-node dissection range from 0 to 1·4%, with fairly short follow-up of 3 years or less.[71] Any residual disease within the axillary nodes will be low volume, and longer follow-up might be needed for any clinical manifestation of regional recurrence. Kujit and Roumen[72] report an actuarial rate of 5% at a median follow-up of 6·5 years, predicting that up to 10% of patients might eventually develop isolated axillary recurrence after a negative sentinel-lymph-node biopsy.

Radiotherapy

Long-term follow-up of breast-conservation trials confirm significantly increased rates of local relapse when radiotherapy is omitted.[46,73] However, rates of IBTR are acceptable when breast-conservation surgery is combined with whole-breast irradiation, usually delivered via conventional tangential breast fields at a total dose of 46–50 Gy in 25 fractions over 5 weeks with an optional booster dose (10–20 Gy). Within the NSABP B-06 trial, 39·2% of patients undergoing wide local excision only had developed local recurrence at 20 years' follow-up compared with 14·3% for those receiving radiotherapy after lumpectomy ($p<0·001$).[46] Moreover, cosmetic results are satisfactory when neither the volume of breast tissue excised or the radiation fraction size are excessive.[56] A group of patients for whom rates of IBTR are not further reduced by radiotherapy compared with observation or tamoxifen therapy alone has not been defined.[74–76] Omission of radiotherapy should be cautioned at present since it can lead to rates of IBTR approaching 30% for small tumours of favourable grade, and local control does affect overall survival.[49] Older women benefit in terms of breast-cancer-specific survival from radiotherapy after breast-conservation surgery, and tamoxifen alone cannot substitute for radiotherapy. Comorbidities can otherwise reduce life expectancy for some older women for whom any additional local control (3–6% risk absolute reduction) from radiotherapy might not be clinically significant.[77,78]

A group of techniques has been developed—accelerated partial-breast irradiation—that decrease the volume of breast tissue irradiated and the duration of treatment. More than three-quarters of true breast recurrences occur at the site of lumpectomy, and whole-breast irradiation might be unnecessary. These techniques are focused on the tumour bed and a zone of surrounding tissue of variable depth. The advent of CT-based treatment planning kept exposure of normal tissues to a minimum and helped radiotherapists cope with the challenges resulting from the peculiar shape of the breast and contiguity of important surrounding structures (eg, heart and lungs). Computer technology assisted with placement of multiplanar interstitial catheter implants for brachytherapy after lumpectomy. Treatment was aimed at the tumour bed and a margin of tissue to a depth of 1–2 cm. This technique allowed radiotherapy (34 Gy) to be completed within 1 week rather than 5 or 6 weeks. Single institution series with more than 5 years of follow-up show rates of local control to be similar to whole-breast irradiation for matched and appropriately selected subsets of patients.[79–81] Despite a US multicentre trial confirming reproducibility of these favourable results across institutions, the perceived complexity of brachytherapy detracted from its popularity and it remains available in only a few centres worldwide.[82]

Two further techniques of accelerated partial-breast irradiation have been pioneered: intraoperative radiotherapy and MammoSite. Intraoperative radiotherapy delivers a high dose of radiation as one fraction at the time of surgery, allowing precise application of radiation dose to the target area to eliminate tumour foci around the surgical bed. It potentially intensifies the tumour kill effect of surgery and radiotherapy, although some are concerned about the radiobiological equivalence of one dose of intraoperative radiotherapy (21 Gy) compared with conventional whole-breast irradiation. This concern applies particularly to the low-energy X-ray source (50 kV) used in the TARGIT trial[83] compared with electron beam therapy (electron intraoperative therapy)[84] for which depth of penetration is restricted. However, mathematical and laboratory models suggest that TARGIT might be better than conventional therapy,[85,86] and initial clinical results are encouraging.[83] Intraoperative radiotherapy facilitates an integrated approach to the multidisciplinary treatment of cancer, but requires specialised equipment and for electron intraoperative therapy a dedicated suite. An alternative technique of brachytherapy is given via a double lumen balloon catheter (MammoSite) placed within the surgical cavity.[87] This device delivers a total dose of 34 Gy in ten fractions (via a high-dose rate remote afterloader) and is now a common method of accelerated partial-breast irradiation, using equipment already present in many centres. Preliminary results with 5 years' follow-up show low rates of local recurrence (0–6%) and good to excellent cosmetic results in 80% of patients.[88,89]

Three-dimesional conformal radiotherapy is a form of accelerated partial-breast irradiation that uses an external beam to treat smaller volumes than whole-breast irradiation does. Despite being non-invasive, achieving high levels of conformality is difficult.[90,91] The related technique of intensity-modulated radiotherapy delivers conformal dose distributions and improves homogeneity.[92] The combined NSABP B-39/RTOG 0413 trial incorporates three techniques for accelerated partial-breast irradiation (three-dimensional conformal, interstitial brachytherapy, and MammoSite) and aims to assess these techniques in comparison with whole-breast irradiation with primary endpoints of local recurrence, disease-free survival, and overall survival.[93] The START trial has assessed accelerated hypofractionated whole-breast irradiation and showed that patients given a lower overall radiotherapy dose in fewer, larger fractions have similar local control and fewer adverse side-effects than does a dose of 50 Gy in a standard 5-week schedule. This finding supports hypofractionation as a safe and effective approach, but long-term follow-up is required to assess local control and late toxic effects.[94]

Radiotherapy after mastectomy encompasses irradiation of the chest wall and skin together with regional lymph nodes. The indications for this treatment continue to evolve, but all trials have shown that it reduces the proportional risk of local failure by two-thirds to three-quarters of patients, including those with tumours larger than 5 cm and four or more positive axillary lymph nodes. However, data for the benefit of postmastectomy radiotherapy in terms of overall survival are conflicting; an overview by the EBCTCG confirms that postmastectomy radiotherapy in node-positive women results in an absolute survival gain at 15 years.[49] Although postmastectomy radiotherapy reduced rates of local relapse in node-negative patients, mortality was not reduced. In the Danish and British Columbia trials of postmastectomy radiotherapy in premenopausal node-positive women receiving chemotherapy,[61,62] the proportional survival benefits were similar for patients with one to three and four or more positive nodes. However, some aspects of trial design were controversial and any survival advantage within the intermediate-risk groups (one–three nodes positive) could be masked by toxic effects from the radiotherapy. New radiation techniques using tangential fields minimise cardiotoxicity, as do methods such as intensity-modulated radiotherapy.[95] The SUPREMO trial is assessing whether modern chemotherapy regimens and postmastectomy radiotherapy can lead to overall-survival improvements in this intermediate-risk group.[96]

Adjuvant systemic therapies

Incorporation of adjuvant systemic therapies into the multidisciplinary management of breast cancer has led to improvements in rates of disease-free and overall survival.[97,98] The indication for adjuvant systemic therapy after definitive surgery is based on established prognostic factors, including age, comorbidities, axillary-lymph-node involvement, tumour size, and tumour grade.[99] In addition to these well established clinicopathological factors, molecular tests can assess the estimated risk of recurrence in patients with early-stage breast cancer and identify distinct biological classes of tumour. Three prognostic tests have been approved for clinical application in the USA:[100] Oncotype DX, MammaPrint, and H/I, which are based on a 21-gene profile, a 70-gene profile, and expression of the *HOXB13/IL17BR* genes, respectively. The Oncotype DX and *HOXB13-IL17BR* assays measure gene expression with reverse-transcriptase PCR and the MammaPrint assay uses complementary DNA microarray technology. One of the advantages of reverse-transcriptase PCR is that gene expression can be measured in formalin-fixed paraffin-embedded tumour tissue, whereas the microarrays need fresh frozen tissue. Although prognostic tests provide information about risk of recurrence and death, predictive markers are needed to select the optimum therapy for individual patients. The best characterised molecular predictive markers are the oestrogen receptor, the progesterone receptor, and the human epidermal growth factor receptor 2 (HER2).[101]

The responsiveness of breast tumours to hormonal manipulation provides a unique therapeutic opportunity in the form of targeted treatment. The antioestrogen tamoxifen confers a proportional reduction in mortality of 26% and up to 47% reduction in local recurrence at 10 years' follow up, with benefit confined to oestrogen-receptor-positive tumours.[98] Tamoxifen is effective in both premenopausal and postmenopausal women, although premenopausal women are eligible for ovarian suppression with either luteinising hormone-releasing hormone analogues or laparoscopic oophorectomy when disease is hormone responsive.[102] An advantage of luteinising hormone-releasing hormone agonists is their potentially reversible effects on cessation of treatment. Whether ovarian suppression can provide an alternative to chemotherapy in patients with oestrogen-receptor-positive disease, and whether luteinising hormone-releasing hormone agonists confer any additional benefit when combined with standard treatments, is being investigated.

The aromatase inhibitors represent an important advance in endocrine therapy of breast cancer. The oral agents anastrozole, letrozole, and exemestane are of comparable antitumour efficacy and are potentially interchangeable, although long-term data for side-effect profiles must be obtained before definitive pronouncements on clinical use. The American Society of Clinical Oncology Technology Assessment recommends that adjuvant hormonal therapy for postmenopausal women should include an aromatase inhibitor prescribed either as initial therapy or sequenced after tamoxifen for 2–3 years' (early switch) or 5 years' duration.[103] The largest study of adjuvant aromatase inhibitors showed a continuing divergence of the curves for disease-free survival at 68 months' follow-up, with evidence of a carry-over effect and a reduction in time to distant recurrence favouring anastrozole.[104] The absolute benefit for time to recurrence has increased from 2·8% at 5 years to 4·8% at 100 months. This head-to-head comparison of tamoxifen versus anastrozole shows no difference in overall survival, although there is a non-significant trend for improved breast-cancer-specific survival in the latest analysis.[105] The fairly good prognostic parameters might ultimately obscure translation into a significant benefit for this endpoint.[104,106]

The IES trial is the only adjuvant study to show an overall-survival advantage for use of an aromatase inhibitor within the conventional 5-year treatment span.[107] These results, together with a meta-analysis of the Austrian (ABCSG 8/ARNO 95) and Italian (ITA) studies, support early sequencing with a switch to an aromatase inhibitor after 2–3 years of tamoxifen[108] as an efficacious approach, with improvement in overall survival for oestrogen-receptor-positive patients. The proportional risk reductions for disease-free survival are greater within the early switch than in head-to-head comparisons of tamoxifen and an aromatase inhibitor. Although interim results of the BIG 1-98 study[109] showed a disease-free survival benefit for 5 years of letrozole compared with 5 years of tamoxifen, definitive results of this study have not shown a clear advantage from an early switch policy in terms of recurrence rates compared with 5 years of an aromatase inhibitor.[110] At a median follow-up of 72 months, there was no significant difference in disease-free survival for 5 years of letrozole compared with either of the switch groups. However, pair-wise comparisons suggested a minor benefit for letrozole (5 years) compared with tamoxifen for 2 years followed by letrozole for 3 years. The inverse sequence of tamoxifen after 2 years of letrozole was equivalent to monotherapy, and patient cross-over from tamoxifen to letrozole precluded any updated comparison of the monotherapy groups.

On the basis of these data, some authorities recommend that patients at greatest risk of relapse might benefit most from an upfront aromatase inhibitor, whereas those with lower hazard rates for relapse might be best treated with an early-switch regimen involving tamoxifen for 2–3 years followed by an aromatase inhibitor for a total duration of 5 years. Although results of the BIG 1-98 study show greatest benefit from aromatase inhibitors for node-positive patients, the converse is true for the ATAC study.[105] Moreover, high-grade tumours derive no additional benefit from upfront aromatase inhibitors, and no conclusive evidence supports HER2 status as being predictive of response to aromatase inhibitors. Benefits in terms of disease-free and overall survival must be balanced against long-term adverse effects on bone health, cognitive function, and cost. Some patients at very low risk of relapse might derive minimal additional benefit from incorporation of an aromatase inhibitor into their treatment schedule and should receive tamoxifen only. Breast-cancer patients remain at chronic risk of relapse, and aromatase inhibitors offer the opportunity for extended adjuvant therapy beyond 5 years with use of an agent with a different mechanism of action. In the MA-17 trial of extended adjuvant treatment,[111] letrozole therapy significantly improved disease-free survival compared with placebo after completion of 5-years' standard tamoxifen treatment in node-positive patients.

Adjuvant chemotherapy improves rates of disease-free and overall survival for patients with early-stage breast cancer irrespective of nodal status.[112] US guidelines recommend adjuvant chemotherapy for healthy patients with axillary-node involvement and for node-negative disease when tumours are larger than 1 cm or in the presence of other adverse prognosticators (eg, age <35 years, negative oestrogen-receptor or progesterone-receptor status, high-grade tumour).[113] All patients within a subgroup are assumed to derive similar benefit from chemotherapy, but many are overtreated and do not have micrometastatic disease at presentation. Identification of patients with distant microscopic spread is particularly relevant in patients with node-negative, oestrogen-receptor

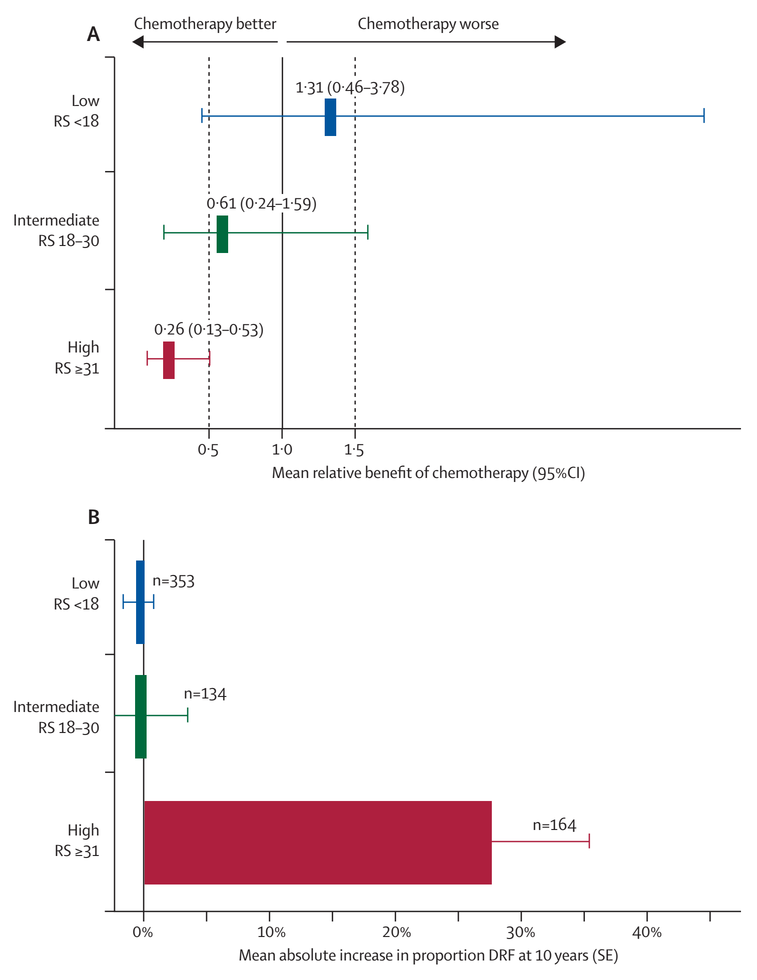

***Figure 4:* Relative (A) and absolute (B) benefit of chemotherapy as a function of recurrence-score (RS) risk category in low, intermediate, and high RS groups**

DRF=distant recurrence free. Reprinted with permission from *Journal of Clinical Oncology*.[115]

or progesterone-receptor-positive breast cancer. Adjuvant endocrine therapy is highly effective in such cases and the contribution of chemotherapy not well defined. The prognostic and predictive roles of the Oncotype DX assay were assessed in archival tissue from treated node-negative, oestrogen-receptor-positive tumours. Tamoxifen was most effective when the recurrence score was low (≤18),[114] whereas patients whose primary tumours had a high (≥31) recurrence score derived more benefit from adjuvant chemotherapy (figure 4).

The 21-gene assay was also predictive of benefit from adjuvant chemotherapy in patients with node-positive breast cancer. A US trial randomly assigned premenopausal women with node-positive, oestrogen-receptor-positive breast cancer to tamoxifen or cyclophosphamide, doxorubicin, and fluorouracil (CAF) before or concurrently with tamoxifen. The sequential use of CAF followed by tamoxifen maximised rates of disease-free survival. However, when the Oncotype DX assay was applied to archival primary tissue, only patients with a high recurrence score benefited from CAF chemotherapy.[116] If prospective clinical trials confirm these data, we might be able to spare patients with low-risk breast cancer from undergoing chemotherapy, irrespective of the size of their primary tumours and degree of nodal involvement. Prognostic and predictive values of these molecular assays are based on retrospective subset analyses;[117] TAILORx is a prospective trial that randomises patients with oestrogen-receptor-positive node-negative breast cancer with an intermediate recurrence score (11–25) to chemotherapy and hormonal therapy, or hormonal therapy alone.[118]

The anthracyclines and taxanes are considered the most effective chemotherapies in the adjuvant setting. The taxanes (paclitaxel and docetaxel) have non-cross resistance with conventional agents, and their mechanism of action is to stabilise and prevent disaggregation of microtubules with disruption of the mitotic spindle. A randomised trial showed that four cycles of doxorubicin/cyclophosphamide (AC) followed by four cycles of paclitaxel improves survival compared with AC alone in patients with node-positive breast cancer.[119] Furthermore, giving AC and paclitaxel every 2 weeks (so-called dose-dense approach) improves disease-free survival compared with administration every 3 weeks.[120] Two trials investigating the efficacy of docetaxel in node-positive patients using more intensive anthracycline regimens noted significant improvements in overall survival for taxane-containing regimens.[115,121] A large trial randomised patients with node-positive breast cancer to four cycles of AC followed by either paclitaxel every 3 weeks, weekly paclitaxel, docetaxel every 3 weeks, or weekly docetaxel. Results showed that four cycles of AC followed by one dose of paclitaxel every week for 12 weeks improved overall survival.[122] Whether six or eight cycles are best, or four are sufficient, is unclear. Efforts are ongoing to identify gene-expression profiles that would help select patients for specific chemotherapies.[123] A popular combination is 5-fluorouracil, epirubicin, cyclophosphamide (FEC)-docetaxel for patients with involvement of four or more nodes. Provisional results from the TACT I trial suggest that adding four cycles of docetaxel to one of two standard regimens containing antracycline for unselected patients has little benefit.[124] Retrospective studies indicate that aberrant HER2 expression could correlate with benefit from paclitaxel in the adjuvant setting[125] and that modulation of topoisomerase II gene expression due to deletion or amplification might predict response to anthracycline-based chemotherapy.[126] Co-amplification of HER2 and the topoisomerase II amplicon is associated with increased response rates to anthracyclines.[126]

Trastuzumab (Herceptin) is a monoclonal antibody directed against the extracellular domain of HER2—a tyrosine kinase involved in cell growth and proliferation. Amplification of the *HER2* gene or otherwise overexpression of the cell-surface protein has been associated with a poor prognosis.[127] The HER2 status of the primary tumour or metastatic deposit should be assessed in all patients with breast cancer with either immunohistochemistry, fluorescence in-situ hybridisation, or chromogenic in-situ hybridisation.[128] If the tumour is HER2 positive, the patient is a good candidate for trastuzumab and for participation in clinical trials of novel HER2-directed treatments.

In patients with HER2-positive early-stage breast cancer, trastuzumab improves rates of disease-free and overall survival independent of age, axillary node metastases, and oestrogen-receptor or progesterone-receptor status.[129–131] Two US trials reported a significant reduction in risk of recurrence of about 50% and showed an early survival benefit favouring trastuzumab at 2 years ($p=0\cdot015$).[129] The European trial (HERA) showed similar reductions in risk of recurrence but no overall-survival advantage.[130] Within the US trials, trastuzumab was given concurrently with an anthracycline-based chemotherapy (AC followed by paclitaxel/trastuzumab) and thereafter continued as single agent therapy for 52 weeks; however, Herceptin was prescribed only after completion of all chemotherapy (any regimen of ≥four cycles) in the European trial. A further international study (BCIRG 006) showed significant improvement in disease-free and overall survival for the non-anthracycline regimen TCH (docetaxel, carboplatin, and trastuzumab) compared with AC-T (doxorubicin/cyclophosphamide followed by docetaxel) and five-fold lower cardiotoxicity than with AC followed by docetaxel/trastuzumab (AC-TH).[131] The risk of cardiac toxicity in the adjuvant setting ranges from 0·5% to 4%.[132] Patients should undergo a baseline echocardiogram or cardiac scan to assess left ventricular ejection fraction before initiation of trastuzumab-based therapy. Serial assessments of left ventricular ejection fraction are recommended every 3 months while receiving trastuzumab with close follow-up in the first 2 years after completion of treatment. In the event of cardiac toxicity, trastuzumab should be discontinued and left ventricular ejection fraction re-assessed in 4 weeks, although this decision should be made on an individual basis and consider recurrence risk and pre-existing cardiac morbidity.

Lapatinib (Tykerb) is a reversible small-molecule tyrosine-kinase inhibitor directed against epidermal growth factor receptor and HER2.[133] The combination of lapatinib and capecitabine improved rates of disease-free survival compared with capecitabine alone in heavily pretreated patients with metastatic breast cancer.[134] Inhibition of the HER2 kinase seems an important target for this type of molecule, because agents that target the epidermal growth factor receptor kinase selectively (eg, gefitinib and erlotinib) have shown insufficient efficacy in unselected patients with metastatic breast cancer.

Pertuzumab is a monoclonal antibody directed against HER2 that prevents formation of heterodimers between HER2 and other members of the HER family.[135] The binding sites of trastuzumab and pertuzumab localise to different domains of the HER2 protein. Preclinical studies showed a synergistic interaction between pertuzumab and trastuzumab, which is being explored.[135] Overexpression of insulin-like growth factor 1 receptor (IGF-IR) is associated with a poor prognosis and resistance to several drugs, including endocrine therapy and trastuzumab.[136] Approaches to inhibit IGF-IR include the use of monoclonal antibodies, small-molecule tyrosine-kinase inhibitors, and IGF binding proteins. Intracellular transduction pathways activated by growth factor receptors such as HER2 and IGF-IR are potential therapeutic targets, and the mitogen-activated protein kinase (MAPK) and the PI3K/Akt/mTOR pathways have been well characterised in breast-cancer cells.[137] Clinical trials are testing several inhibitors directed against different aspects of these signalling pathways. Postreceptor signalling pathways are not linear but form complex networks with much crosstalk. Multiple compensatory mechanisms exist with some functional redundancy, and blocking one protein (eg, mTOR) often leads to activation of more proximal steps (eg, Akt) and potentially increased proliferation.[137] An approach for overcoming the compensatory loops is to use a combination of inhibitors and to target central signalling nodes that are crucial for sustained growth-inhibitory effects. Novel approaches target not only the cancer cells but also the tumour microenvironment and new vessel formation. Preclinical and clinical studies have shown that blocking angiogenesis improves the efficacy of cytotoxic chemotherapy. Bevacizumab is a recombinant, humanised monoclonal antibody to vascular endothelial growth factor. A phase II trial of trastuzumab and bevacizumab showed that this combination was highly effective in patients with HER2 overexpressing metastatic breast cancer who had failed previous therapies.[138] Randomised clinical trials are in progress to establish the safety and efficacy of bevacizumab in combination with chemotherapy, endocrine therapy, and trastuzumab in all subtypes of breast cancer.

Primary systemic therapies

Primary systemic therapy, also known as neoadjuvant or preoperative therapy, was initially used for management of locally advanced breast cancers that could be rendered technically operable. Neoadjuvant approaches have increasingly been championed for treatment of operable tumours, with the expectation of improved outcomes and possible breast-conservation surgery.[139,140] Downstaging might reduce the requirement for mastectomy by up to half, and breast-conservation surgery is more likely for unifocal tumours located away from the nipple areolar complex.[141,142] Since the primary tumour remains in situ, primary systemic therapy allows serial core biopsies to be undertaken with monitoring of treatment effects. Primary systemic therapy constitutes a powerful in-vivo model providing potential information about pathological and molecular predictors of response and tumour biology, which in conjunction with imaging parameters, enables non-responders to be identified early and therapy changed accordingly.

Early trials of primary systemic therapy compared the same schedule of chemotherapy before or after standard surgical treatment. The NSABP B-18 trial randomised patients to four cycles of anthracycline-based chemotherapy before or after surgery. Overall survival was equivalent for both approaches, but patients receiving primary systemic therapy were more likely to undergo breast-conservation surgery.[141,142] However, rates of IBTR were higher when surgery followed rather than preceded chemotherapy, but this difference did not reach statistical significance (10·7% *vs* 7·6%, p=0·12).[142] Patients with a complete pathological response to primary systemic therapy have improved disease-free and overall survival, suggesting its use as a surrogate marker for trials comparing different schedules of primary systemic therapy.[141,143–145] The next generation of trials of primary systemic therapy aimed to establish whether different preoperative regimens could improve outcomes. In a small randomised trial, addition of docetaxel to a preoperative schedule compared with further anthracycline drugs doubled the complete pathological response with lengthening of disease-free and overall survival at 3 years.[146] By contrast, NSABP B-27 confirmed a doubling of the complete pathological response with addition of four cycles of docetaxel to four cycles of AC, but no improvement in overall survival.[147] Nonetheless, NSABP B-27 did show improved outcomes for patients who achieved a complete pathological response irrespective of schedule received.[147] Unlike adjuvant therapy trials, fewer numbers of patients and shorter follow-up are needed for assessment of primary systemic therapy. The activity of trastuzumab in the adjuvant setting has confirmed the benefits of this agent when combined with chemotherapy in the neoadjuvant setting; complete pathological response rate more than doubled for combined therapy versus chemotherapy alone.[148] However, the potential for omission of surgical resection in patients with a complete pathological response remains limited in the absence of good clinico-radio-pathological correlation and prospective identification of this subset with imaging and percutaneous biopsy.

Oestrogen-receptor-negative tumours have higher rates of complete pathological response to primary chemotherapy than do hormone-sensitive tumours that exhibit lower rates.[147–150] Material from core biopsies or fine-needle aspirates can be processed for construction of DNA microarrays, allowing comparison of expression profiles between responders and non-responders.[151,152] A meta-analysis of neoadjuvant versus adjuvant systemic therapy for early-stage breast cancer shows that disease-free and overall survival are comparable for the two schedules.[153] Even if surgery causes a systemic perturbation that can be offset by induction chemotherapy, the assumption that a modest shift in the timing of chemotherapy relative to surgery would have any significant clinical effect is perhaps naive.[154] Moreover, increased rates of IBTR for neoadjuvant regimens could suggest inadequate surgery and cast doubt on the model of downstaging to allow breast-conservation surgery.

By analogy with neoadjuvant chemotherapy, hormonal treatment can be used preoperatively to downstage tumours. Hormonal therapies are less toxic and potential side-effects of chemotherapy can be avoided in elderly receptor-positive-patients and those with a poor performance status.[155] Moreover, oestrogen-receptor-positive patients are less likely to achieve a complete pathological response with primary chemotherapy than oestrogen-receptor-negative patients are.[147,149,150] Most studies relate to postmenopausal women for whom the aromatase inhibitors have consistently outperformed tamoxifen in the neoadjuvant setting, when endpoints include response rates and breast-conservation rates.[156,157] The amount of oestrogen-receptor expression is the main determinant of response, and the optimum duration of therapy might be longer than for chemotherapy.[158] In a study of patients with hormonally-sensitive locally-advanced and inoperable breast cancer, letrozole given as a preoperative schedule for 4 months yielded significantly better response rates than did tamoxifen for a similar period and allowed a significantly higher rate of breast-conservation surgery.[156] The IMPACT study randomised patients with operable hormone-responsive breast cancer to 3 months of anastrozole alone, tamoxifen alone, or a combination of the two. No significant difference in objective clinical response rates were recorded, but anastrozole was more effective than tamoxifen was in reducing Ki-67 expression and downstaging tumours to allow breast-conservation surgery according to surgeon assessment.[157]

Tumours do not necessarily shrink in a concentric manner in response to chemotherapy. Even if no viable cancer cells remain at the site of the original tumour periphery, this zone might contain unstable epithelium that is prone to malignant change.[159] Furthermore, tumour regression is difficult to assess radiologically even with MRI.[160] Functional imaging techniques have a potential role in assessment of disease extent before and after chemotherapy and can be especially useful in early assessment of tumour response as changes in metabolism, cell proliferation, and vascularity precede tumour regression.[161]

Primary systemic therapy can cause differential downstaging between sentinel and non-sentinel lymph nodes.[162] Biopsy of sentinel lymph nodes undertaken before chemotherapy will keep the risk of a false-negative result to a minimum and ensure that decisions for postmastectomy radiotherapy are based on accurate nodal staging.[163] However, there is no quantification of regional metastatic load, and some advocate biopsy of sentinel lymph nodes after primary systemic therapy to take advantage of nodal downstaging and avoidance of axillary dissection in up to 40% of patients.[162]

Chemoprevention

Tamoxifen is a pioneering non-steroidal antioestrogen whose primary action is to competitively antagonise oestrogen at the cellular-receptor level.[164] It has a proven efficacy in treatment of breast cancer over the past 30 years,[165] with substantial increases in survival in patients receiving long-term adjuvant therapy.[166] Furthermore, patients receiving adjuvant tamoxifen have a 47% reduction of contralateral tumours.[167] This accrual of a vast clinical database, underpinned by data from preclinical models and in-vitro studies,[168] catalysed the exploration of tamoxifen as a chemopreventive in high-risk women.[169] Several placebo-controlled chemoprevention trials of tamoxifen in high-risk premenopausal and postmenopausal women have shown up to a 50% reduction in the cumulative incidence of both invasive and non-invasive breast cancer, with primary effects confined to oestrogen-receptor-positive disease.[170,171] Moreover, recent data suggest that not only is tamoxifen effective during therapy, but also that chemoprevention is enhanced for many years after treatment ends.[171–173] This important observation shows the continuing antitumour action of tamoxifen, which occurs at a time when there are very few side-effects from the drug. The side-effect profile of tamoxifen and other potential agents are crucial considerations in the chemopreventive setting when the risk to benefit ratio is shifted and healthy women receive a pharmacological intervention for which the benefits are less tangible.

A modest increase in endometrial cancer in postmenopausal women has been well documented,[171] although neither this increase nor a raised risk of thromboembolism has been noted in premenopausal women.[171] Tamoxifen can result in hot flushes when used as adjuvant therapy in patients with breast cancer and is a potential cause of non-compliance. This side-effect is especially pertinent in women who are considering use of tamoxifen for chemoprevention, and a selective serotonin reuptake inhibitor often needs to be co-prescribed. However, the latter can interfere with conversion of tamoxifen to its active metabolite endoxifen and should not be used;[174] the occurrence of hot flushes can indicate effective metabolism.[175] Additionally, mutations in *CYP2D6* can impede conversion of tamoxifen to endoxifen and might be relevant to patients considering use of tamoxifen for chemoprevention.

Concerns over increased incidence of endometrial cancer in women taking tamoxifen have led to re-assessment of other non-steroidal antioestrogens with attenuated uterotrophic activity in the rodent uterus.[176] The recognition that non-steroidal antioestrogens such as tamoxifen and raloxifene were selective oestrogen-receptor modulators with duality of action created a new dimension in therapeutics that is being exploited in chemoprevention strategies. If a selective oestrogen-receptor modulator is oestrogenic in bone but antioestrogenic in breast tissue, then perhaps it could be used to prevent osteoporosis with concomitant prophylaxis of breast cancer in post-menopausal women.[177] Raloxifene has been successfully tested for reduction of fractures in women at high risk for osteoporosis[178] and significantly reduces the incidence of oestrogen-receptor-positive breast cancer (77% risk reduction) in patients receiving long-term raloxifene for prevention of this disease.[179] These encouraging findings combined with the desire to minimise side-effects sporned the STAR trial:[180] a head-to-head comparison of tamoxifen and raloxifene as chemopreventive agents in high-risk postmenopausal women. Initial results have shown that raloxifene is equivalent to tamoxifen in reducing the incidence of oestrogen-receptor-positive breast cancer by 50%, but is less effective in prevention of non-invasive disease.[180] Raloxifene might therefore interfere with the progression of in-situ to invasive disease, but have no effect on premalignant to in-situ transition. Raloxifene had a more favourable side-effect profile than did tamoxifen, with marginally significant reductions of thromboembolic events, cataracts, lens replacement, and endometrial cancer.

The panel summarises US recommendations for use of selective oestrogen-receptor modulators on the basis of clinical-trial evidence and approvals from the Food and Drug Administration for risk reduction.[171,181] Recommendations for treatment duration are a single pulse of 5 years. However, the long-term use of raloxifene to prevent osteoporosis could mandate 10 or more years of therapy, but the apparent carry-over effect maintains the antitumour efficacy of raloxifene and tamoxifen beyond the actual treatment period.[180]

Future research should be directed at elucidating the mechanism of action of selective oestrogen-receptor modulators in different target tissues around the body. The configuration of the ligand/oestrogen receptor complex determines the recruitment of co-activators and co-repressors that bind to the external surface of the complex and activate oestrogen-response elements.[182] Individual selective oestrogen-receptor modulators have a clinical signature, with a range of structure-activity profiles that are site specific and confer differential and non-correlative mixed agonist or antagonist activity between species and tissues. Finally, clinical trials are assessing aromatase inhibitors in high-risk postmenopausal women as chemopreventive agents. IBIS II is a multicentre trial[183] that randomises healthy women at increased risk of breast cancer to either anastrozole or placebo. These inhibitors are associated with a greater reduction of contralateral breast cancer in adjuvant trials than is tamoxifen. They could potentially be combined with an luteinising hormone-releasing hormone agonist as a chemopreventive strategy in premenopausal women, but concerns exist about side-effects of profound oestrogen deprivation, and the optimum duration of therapy is unknown.

Panel: US recommendations for use of selective oestrogen-receptor modulators

Tamoxifen

Recommended for high-risk premenopausal women for whom there is no significantly increased risk of endometrial cancer or blood clots

Raloxifene

Recommended for high-risk postmenopausal women for whom there is no significantly increased risk of endometrial cancer

Raloxifene

Recommended for treatment and prevention of osteoporosis. It reduces the risk of breast cancer with no increased risk of endometrial cancer

Conclusions

Despite an inexorable rise in the incidence of breast cancer, improvements in treatments together with screening have led to modest falls in mortality. Local control of disease does affect overall survival, and greater attention to surgical margins and improved radiotherapy techniques have reduced local recurrence after breast-conservation surgery. Oncoplastic surgical techniques are being used selectively to enhance cosmetic outcomes while satisfying oncological mandates. Long-term outcome is determined by the presence and behaviour of distant micrometastases, which have to be effectively managed to achieve disease control if not cure. Molecular profiling offers the potential to provide predictive information about individual tumour response, which will guide clinical application of targeted biological therapies and rationalise their integration with conventional systemic treatments.

Conflicts of interest

We declare that we have no conflicts of interest.

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THE PARADOX OF OESTRADIOL-INDUCED BREAST CANCER CELL GROWTH AND APOPTOSIS

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Abstract

High dose oestrogen therapy was used as a treatment for postmenopausal patients with breast cancer from the 1950s until the introduction of the safer antioestrogen, tamoxifen in the 1970s. The anti-tumour mechanism of high dose oestrogen therapy remained unknown. There was no enthusiasm to study these signal transduction pathways as oestrogen therapy has almost completely been eliminated from the treatment paradigm. Current use of tamoxifen and the aromatase inhibitors seek to create oestrogen deprivation that prevents the growth of oestrogen stimulated oestrogen receptor (ER) positive breast cancer cells. However, acquired resistance to antihormonal therapy does occur, but it is through investigation of laboratory models that a vulnerability of the cancer cell has been discovered and is being investigated to provide new opportunities in therapy with the potential for discovering new cancer-specific apoptotic drugs. Laboratory models of resistance to raloxifene and tamoxifen, the selective oestrogen receptor modulators (SERMs) and aromatase inhibitors demonstrate an evolution of drug resistance so that after many years of oestrogen deprivation, the ER positive cancer cell reconfigures the survival signal transduction pathways so oestrogen now becomes an apoptotic trigger rather than a survival signal. Current efforts are evaluating the mechanisms of oestrogen-induced apoptosis and how this new biology of oestrogen action can be amplified and enhanced, thereby increasing the value of this therapeutic opportunity for the treatment of breast cancer. Several synergistic approaches to therapeutic enhancement are being advanced which involve drug combinations to impair survival signaling with the use of specific agents and to impair bcl-2 that protects the cancer cell from apoptosis. We highlight the historical understanding of oestrogen's role in cell survival and death and specifically illustrate the progress that has been made in the last five years to understand the mechanisms of oestrogen-induced apoptosis. There are opportunities to

harness knowledge from this new signal transduction pathway to discover the precise mechanism of this oestrogen-induced apoptotic trigger. Indeed, the new biology of oestrogen action also has significance for understanding the physiology of bone remodeling. Thus, the pathway has a broad appeal in both physiology and cancer research.

Historical origins of targeted hormonal therapy

the In 1908 Paul Ehrlich received the Nobel Prize for Physiology and Medicine for his work on targeted therapy. His concept was based on the idea that antibodies could bind to specific molecular targets associated with disease, but remain non-toxic to healthy tissue [1]. Although he did not live long enough to receive a second Nobel Prize (nominated in 1914), Ehrlich's laboratory also devised a practical method of chemical therapy (or chemotherapy) to treat disease. Organic compounds were synthesized and tested in laboratory animal models of the disease to be treated, and then promising compounds were tested in patients. The result was arsphenamine, also known as Salvarsan for the treatment of syphilis. This was first successful treatment of any disease by a synthetic drug. His method is used to this day, however his efforts to apply the concept to cancer were not successful. In the year of his death in 1915 Ehrlich declared "I have wasted 15 years of my life in experimental cancer research"[2]. Nevertheless, Ehrlich's vision triggered an extensive search for specific drugs that would target tumours.

In 1896 Beatson [3] published his findings of the beneficial effects of oophorectomy in premenopausal patients with advanced breast cancer. Beatson had based his approach on the role of the ovaries in mammalian lactation, and presumed that there would be a similar mechanism for breast cancer growth. The successful treatment of breast cancer by oophorectomy indicated that there was a principle in the ovary that travels around the body to control the growth of a cancer. The mechanism, however, was to remain unknown until 1923 when Allen and Doisy [4] showed that there is an ovarian hormone that they called oestrogen, which caused vaginal cornification and estrus in ovariectomized mice. Their method, employing ovariectomized mice, was subsequently used to test synthetic compounds for oestrogenic activity. Sir Charles Dodds

[5] discovered the potent oestrogenic activity of the stilbenes and he was to discover and define the biological properties of diethylstilboestrol. The oestrogenic properties of triphenylethylene was also described around this time, but in contrast to diethylstilboestrol, triphenylethylene produces long term oestrogenic effects for many weeks [6].

Sir Alexander Haddow was particularly interested in the idea of chemical therapy so he initiated a program to explore the antitumour properties of polycyclic hydrocarbons in laboratory animals. Several compounds caused tumour regression but the fact that the chemicals were carcinogenic prohibited testing in humans. He reasoned that the triphenylethylene based oestrogens had a structural similarity to the polycyclic hydrocarbons and he found they also caused tumour regression in animals. He therefore chose to evaluate the application of oestrogens for the treatment of breast and prostate cancer. In 1944 Haddow [7] published the results of his clinical trial with the synthetic oestrogens triphenylchlorethylene, triphenylmethylethylene, and stilboestrol administered at high doses. He found that 10 out of 22 post-menopausal patients with advanced mammary carcinomas, who were treated with triphenylchlorethylene, had significant regression of tumour growth. Five patients out of 14 who were treated with high dose stilboestrol produced similar responses. Two patients with prostate cancer (out of 30 with diseases other than breast cancer) had a response. The finding that high doses of synthetic oestrogens induced regression of tumour growth in some, but not all patients with breast cancer was somewhat reminiscent of the apparent random responsiveness of oophorectomy in premenopausal patients with metastatic breast cancer [8]. However, Haddow [7] noted that the first successful use of a chemical therapy to treat cancer (breast and prostate) also had significant systemic side effects, such as nausea, areola pigmentation, uterine bleeding, and edema of the lower extremities.

During the 1950's Kennedy [9] systematically investigated the efficacy of synthetic oestrogens for the treatment of advanced breast cancer. Kennedy examined a variety of different oestrogens but there were no significant differences and diethylstilboestrol became the standard drug. However, side effects remained a concern and responses lasted for only about a year in the majority of patients. Anorexia was noted in 57% of cases, nausea in 58%, nipple pigmentation in 80%, vaginal bleeding in 33%, and vomiting in 32% of cases in a large series of 200 patients with advanced breast cancer. By the 1960's, the standards for the endocrine treatment of breast cancer were established. Pre-menopausal women should be treated with ovarian irradiation therapy or bilateral oophorectomy. However, based on data from clinical trials, post-menopausal patients with advanced breast cancer should be treated with high dose of the most potent oestrogenic compound diethylstilboestrol [10]. Overall, one could anticipate that 36 % of patients would respond favorably to high dose oestrogen therapy [11].

The question that needed to be answered if endocrine therapy was to be further advanced was, who to treat? In other words, could one develop a test to predict responsiveness? Even as recently as 1970 Haddow [12] was not enthusiastic about the overall prospects of chemical therapy. He stated that there was unlikely to be a *chemotherapia specifica* in Ehrlich's sense because cancer cells are so similar to normal tissue. Also, unlike the antibiotics where one could pretest responsiveness of the disease to a particular therapy, no such tests existed for cancer. Additionally, it was important that safer less toxic "oestrogens" were developed that might extend therapeutic use. There were clues that deciphering the mysteries of endocrine therapy could be of major benefit for patients. Haddow [13] noted that high dose oestrogen therapy was more successful as a treatment for breast cancer the farther the woman was from the menopause. Oestrogen-withdrawal somehow played a role in sensitizing tumours to the antitumour actions of oestrogen. Later he [12] stated: "In spite of the extremely limited practicality of such measure [high dose oestrogen], the extraordinary extent of tumour regression observed in perhaps 1% of post-menopausal cases has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us". Nevertheless, interest in endocrine therapy waned in the 1970's with the advent of "successful" combinations of cytotoxic chemotherapies for the treatment of metastatic breast cancer. "Coopers Cocktail" of five different chemotherapeutic agents demonstrated dramatic response rates of up to 80% [14]. Cytotoxic chemotherapy was about to become the answer to cancer. But what happened to the triphenylethylene-based oestrogens? Was there another way to improve cancer therapy and to discover the cellular mechanisms of oestrogen action that control the life and death of breast cancer cells?

Non-steroidal Antioestrogens: evolution to targeted therapy.

Leonard Lerner [15] reported the pharmacological properties of the first non-steroidal antioestrogen MER25 or ethamoxytriphetol. The compound was antioestrogenic in all species tested and exhibited no oestrogenic properties. However, the finding that MER25 was a postcoital contraceptive in laboratory animals [16] ignited an intense search of the structure activity relationships by medicinal chemists in the pharmaceutical industry. The goal was to find safer, more potent agents for clinical evaluation. The method chosen for drug discovery was Ehrlich's i.e.: study the structural organic chemistry using the clues provided by the lead compound MER25. The plan of the chemists was simple: place a strategically located alkylaminoethoxy side chain on numerous nonsteroidal oestrogens and then test them as postcoital contraceptives in rats and mice [17]. Although, the oestrogen receptor (ER) had been proposed as the conduit of oestrogen action in its target tissues [18], the actual ER protein was not isolated until 1966 [19]. As a result potential antioestrogens were not screened and identified using an ER assay but drug discovery followed Ehrlich's dictum of a laboratory model *thought* to represent human physiology. When the first clinically useful compound MRL41 or clomiphene was tested in women, it was not a contraceptive, but did the opposite; it induced ovulation. The basic reproductive endocrinology of the rat is completely different than that of a woman. Clomiphene is still used today as a profertility agent in subfertile women [20]. However, clinical trials of clomiphene in the early 1960's did move forward to evaluate activity in the treatment of breast cancer, but were terminated by the company because of concerns about the drug's potential to cause cataracts [20].

The story of the early development of tamoxifen (ICI 46,474) was similar to clomiphene's but the reason that clinical trials were terminated was not because of concerns about toxicity but rather the economic issue of insignificant projected profits that would accrue from marketing a drug for the induction of ovulation and a few thousand patients with metastatic breast cancer, treated for about a year [20].

Arthur Walpole was the leader of the antifertility program at Imperial Chemicals Industry (ICI) Pharmaceuticals (now Astra Zeneca), when ICI 46,474 the pure trans-isomer of a substituted triphenylethylene was discovered and described as a postcoital contraceptive in the rat [21] Earlier in his career [22], Walpole was interested in carcinogenesis and cancer chemotherapy. He also attempted to discover (unsuccessfully) why only some post-menopausal women with metastatic breast cancer respond to high dose oestrogen therapy [23]. It was Walpole who ensured that ICI 46,474 was tested in the clinic and placed on the market as an orphan drug while ICI invested in the scientific research by others in academia to conduct a systematic study of the anticancer actions of tamoxifen and its metabolites [24]. This investment reinvented tamoxifen as the first anticancer agent specifically targeted to the ER in the tumour and created the scientific principles to ultimately establish tamoxifen as the “gold standard” for the adjuvant therapy of breast cancer and as the first chemopreventative agent that reduces the incidence of breast cancer in women with elevated risk [25],[26].

Oestrogen Receptors

The existence of the ER was predicted after Elwood Jensen [18] described the retention of subcutaneously administered tritiated 17β-oestradiol (E2) in target tissues such as uterus and vagina of the immature rats, but not in nontarget tissues such as kidney, liver, and muscle. The actual ER protein was extracted and identified from rat uterus in 1966 by Toft and Gorski [19]. Twenty years later in 1986 the ER gene was cloned [27], but afterwards was renamed as ERα because a second ER was cloned from a rat prostate cDNA based on the sequence similarity to ERα, and called ERβ [28]. Both ERs Fig.(**1**) are members of nuclear hormone receptor superfamily and bind oestrogens with high affinity and regulate transcription of oestrogen responsive genes [29].

The ERα gene is located on chromosome 6q25.1 [30] and encodes a 595 amino acid, 66 kDa protein composed of six domains [31] (Fig.(**1**). The first domain is called the amino-terminal A/B region and contains the ligand-independent and transcriptionally minor activating function-1 (AF-1). The second domain is called the C region that contains the DNA-binding domain (DBD), whose zinc fingers are responsible for ERα’s binding to oestrogen response elements (EREs) found in the promoters of oestrogen responsive genes. The third domain is called the D region, which contains the nuclear localization signal. The fourth domain is called the E region and contains the ligand binding domain (LBD). The ligand-dependent and transcriptionally major activating function-2 (AF-2) is found in the LBD which mediates binding of the coactivators via nuclear receptor boxes composed of LXXLL-like motif [32]. The LBD is composed of 12 α helices, of which helix H3-H12 form a ligand-binding cavity with H12 acting as a “lid” for the cavity. The carboxy-terminal region of the receptor is called the F region. In the nucleus, unliganded monomeric ERα is bound with heat shock proteins (HSPs). Once in the nucleus, oestradiol binds to the LBD of the ERα-HSP complex, and leads to disassociation of HSPs. The LBD then undergoes a crucial conformational change in which H12 caps the ligand binding cavity, and the receptor homodimerizes with another ERα molecule [33]. In addition to capping the LBD, the altered conformation of H12, exposes the coactivator bindings site for coactivators, such as, steroid receptor coactivator 1 (SRC-1), 2 and 3, thus resulting in transcription of oestrogen responsive genes.

The ERβ gene is located on chromosome 14q23.2 and encodes a 530 amino-acid protein. As mentioned above ERα and ERβ share some sequence similarity, in particular they share the highest degree of amino acid sequence, 61 and 97% in LBD and DBD respectively, however A/B and D domains have only 27% and 26% amino acid homology respectively. ERβ is expressed in the testis, prostate, ovary, developing uterus, breast, vascular endothelium, smooth muscle, immune system, bone and some neurons. With lack of homology of A/B domain between ERα and ERβ, functional studies have indicated that ERβ lacks AF-1 activity [34]. However the real significance of ERβ in breast cancer remains unclear [35], and ERα is considered the molecular target for treating and preventing cancer [36], with the SERMs tamoxifen and raloxifene.

Selective Oestrogen Receptor Modulation.

Tamoxifen was originally referred to as a non-steroidal antioestrogen [21]. As more has become known about its molecular pharmacology it has become the pioneering Selective Oestrogen Receptor Modulator (SERM). Tamoxifen was first described as both a partial oestrogen agonist and antagonist in the rat uterus, and a full oestrogen in the mouse uterus and vagina [21]. These were the first important facts which helped to clarify the target-site-specific actions of SERMs. The concept of SERM action was defined by four main pieces of laboratory evidence: 1) ER-positive breast cancer cells inoculated into athymic mice grew into tumours in response to oestradiol, but not to tamoxifen (antioestrogenic action), however both oestradiol and tamoxifen induced uterine weight increase in mice (oestrogen action) [37]; 2) raloxifene (another nonsteroidal antioestrogen), which is less oestrogenic in rat uterus, maintained the bone density in ovariectomized rats (oestrogen action), as did tamoxifen [38], and prevented mammary carcinogenesis (antioestrogenic action) [39]; 3) tamoxifen blocked oestradiol-induced growth of ER-positive breast cancer cells in athymic mice (antioestrogenic action), but induced rapid growth of ER-positive endometrial carcinomas (oestrogenic action) [40]; 4) raloxifene was less effective in promoting endometrial cancer growth (less oestrogenic action) [41]. These laboratory results translated well into clinic where it was shown that tamoxifen effectively can reduce the incidence of breast cancer in high-risk pre- and postmenopausal women, however increases the incidence of blood clots and endometrial cancer, which is linked to oestrogen-like actions of tamoxifen in these tissues in postmenopausal women, who have a low-oestrogen environment [26]. Furthermore, raloxifene maintains bone density in postmenopausal women and reduces fractures [42], but simultaneously reduces the incidence of breast cancer without increasing the incidence of endometrial cancer [43]. In the study of tamoxifen and raloxifene (STAR) both SERMs were equivalent at reducing the incidence of breast cancer in high risk postmenopausal women, but raloxifene appeared to have a safer toxicity profile [44]. With the recognition [45] and effective transition of SERMs to clinical practice, it is now important to understand their mechanism of action so new and novel applications can be developed [46].

Mechanism of SERM Action

The mechanism of SERM action is dependent upon a complex decision network in target tissues to program the cells to express oestrogenic or antioestrogenic actions. There are two ER's: ERα and ERβ and it is possible that a different ratio of both ER's could be important for chemoprevention with SERMs. A high ERα-ERβ ratio correlates with high cellular proliferation; in contrast the low ratio correlates with low cellular proliferation. In other words ERβ tends to suppress cell proliferation and may enhance apoptosis [47]. This is probably because ERα and ERβ have functional differences that can be traced back to differences in the AF1 domain, in particular that they share only 27% of homology in the amino-terminus of the protein. In contrast both ER's differ only by one amino acid in the C region (DNA binding region) and both ER's are able to regulate transcriptional activity of genes regulated by oestrogen response elements (EREs). In this regard ERβ does not have an active AF1 region which is the reason for its inhibitory properties within the cell.

Extensive structure-function relationship studies were initially used to develop a molecular model of oestrogen and antioestrogen action [48-50]. The hypothetical model required the envelopment of a planar oestrogen within the LBD of the ER complex. In contrast, the three-dimensional triphenylethylene binding in the LBD cavity prevents full ER's activation by keeping the LBD open. This structural perturbation of the ER complex is achieved by a correctly positioned bulky alkylaminoethoxy side chain on the SERM. This model was enhanced following studies to solve the X-ray crystallography of the LBD ER's bound with an oestrogen or an antioestrogen. The LBD of ERα is formed by H2-H11 helices and the hairpin β-sheet, while H12, in the agonist bound conformation closes over the LBD cavity filled with E2. The steroid is sealed within the hydrophobic pocket. Oestrogen is aligned in the cavity by hydrogen bonds at both ends of the ligand, particularly the 3-OH group at the A-ring end of E2 forms a hydrogen bond network with Glu353 and Arg394, while E2's 17β-OH group at the D-ring end of the ligand forms a hydrogen bond with ER's His524. This allows hydrophobic van der Waals contacts along the lipophilic rings of E2, in particular between Phe404 and E2's A-ring, to promote a low energy conformation [51]. This results in sealing of the ligand-binding cavity by H12, and exposes the AF-2 surface for interaction with coactivators to promote transcriptional transactivation. In contrast, tamoxifen bound to ER's LBD blocks the closure process by relocating H12 away from the binding pocket, thus preventing coactivator molecules from binding to the appropriate site on the external surface of the complex [52]. Therefore, it is the external shape of the ERs that is being modulated by the ligand which dictates the binding of coactivator molecules. In other words, the shape of the ligand actually causes the receptor to change shape and programs the ER complex to be able to bind coregulator molecules. However, the simple model of a coregulator controlling the biology of an ER complex is not that simple (Fig.(**2**). The modulation of the oestrogen target gene is in fact, regulated by a dynamic process of assembly and destruction of transcription complex at the promoter site of a target gene.

Coregulators and Oestrogen Receptor Action

After ER is bound to an agonist ligand, its conformation changes allowing coregulator molecules to bind to the complex, for example, SRC-3. SRC-3 is a core coactivator that also attracts other coregulators that do not directly bind to ER, such as p300/CBP histone acetyltransferase, CARM1 methyltransferase, and ubiquitin ligases UbC and UbL. All of these coregulators perform specific subreactions within the protein complex of ER and DNA necessary for transcription of target genes, such as chromatin remodeling through methylation and acetylation modifications, and also direct their enzymatic activity towards adjacent factors, which promote dissociation of the coactivator complex and subsequent ubiquitinilation of select components for proteosomal degradation. As a result, this allows the next cycle of coactivator-receptor-DNA interactions to proceed and the binding and degradation of transcription complexes sustain gene transcription (Fig.(**2**).

It is well established that ER is downregulated in the presence of E2 through ubiquitin proteasome pathway. Downregulation is crucial for ER's transcriptional activity. O'Malley [53] used ER positive MCF-7 cells to demonstrate that the 26S proteasome inhibitor MG132 ablated the transcriptional activity of ER, in luciferase activity assays as well as the endogenous transcription of oestrogen responsive genes, such as pS2 or progesterone receptor (PR) gene. Indeed this is a general principle as proteasome-mediated degradation is crucial for other nuclear receptors to function, such as PR and thyroid hormone receptor. The ubiquitin proteasome pathway is responsible for degradation and turnover of a number of transcriptional factors, such as NF-κB, and fos/jun. Through a number of enzymes (ubiquitin-activating [UBA], with which ubiquitin protein forms a high-energy thioester bond, and ubiquitin-conjugating/ubiquitin ligase enzymes), the ubiquitin protein covalently binds to proteins marked for degradation by the 26S proteasome, which subsequently degrades the targeted protein molecules. Lonard and his group [53] have demonstrated that blocking proteosomal degradation with MG132 attenuates the transcriptional activity through both the AF-1 and AF-2 domains, demonstrating that proteasome function is required for efficient transcription through either activation function. At the same time disruption of coactivator binding sites abrogates the ligand-mediated downregulation of the ER.

Armed with the knowledge that oestrogen agonists induce a conformation of the ER that stabilizes coactivator binding, it was logical to ask a question whether the binding of coactivators to ER cause a reciprocal stabilization of agonist ligand binding. This question was answered in studies with use of peptides with sequences derived from coactivator (SRC-1) binding sites on the ER (Nuclear-Receptor boxes), and tetrahydrochrysene-ketone (THC-ketone), DES and E2 as oestrogen antagonists [54]. Overall, coactivator peptides can stabilize the complex between the ER and agonist ligands (E2, DES, THC-ketone), with a marked reduction in ligand dissociation rate from the ligand-receptor complexes. Nevertheless, these coactivator peptides were much less effective in stabilizing ER-antagonist complexes, which was demonstrated in reporter-gene assays, where the elevation of SRC-1 levels increased the potency of E2, it decreased the potency of antioestrogens.

With this brief background of the molecular biology of oestrogenic and antioestrogenic modulation in target tissues we will survey the practical application of this knowledge for the treatment and prevention of breast cancer.

Clinical Applications of SERMs

The clinical application of the laboratory principle of targeting the ER with long-term antihormonal therapy [24] to treat breast cancer has become the standard of care. Two different approaches to adjuvant antihormonal therapy have been developed in the past 30 years: first, is the blockade of oestrogen-stimulated growth [36] at the tumour ERs, and the second one, is the use of aromatase inhibitors to block oestrogen biosynthesis in postmenopausal patients [55]. Aromatase inhibitors have an advantage in the therapy of postmenopausal patients over tamoxifen, firstly, because there are fewer side effects, such as blood clots or endometrial cancer, and aromatase inhibitors have a small, but still significant efficacy in increasing disease free survival [56]. However, most postmenopausal patients worldwide continue to undergo treatment with tamoxifen, either for economic reasons or because they were hysterectomized and also have a low risk of developing blood clots (low body mass index and are athletically active). In premenopausal women, long term tamoxifen is the antihormonal therapy of choice for the treatment of ductal carcinoma in situ (DCIS) [57], the treatment of ER-positive breast cancer [25] and the reduction of breast cancer incidence in those premenopausal women at elevated risk [26]. It is important to stress that premenopausal women treated with tamoxifen do not have elevations in endometrial cancer and blood clots, thus risk: benefit ratio is in favor of tamoxifen treatment [58].

The development of raloxifene from a laboratory concept [59] to an effective clinical strategy to prevent both osteoporosis and breast cancer [43], [44] has opened new opportunities for clinical applications of SERMs. However, the biggest advantage of raloxifene is that it does not increase the incidence of endometrial cancer [44], which was noted in postmenopausal women taking tamoxifen [26].

The current trend is to employ long-term treatment durations to treat disease with SERMs or aromatase inhibitors. Decades of raloxifene must be used to treat and prevent osteoporosis [60]. Additionally longer treatment trials for breast cancer with either aromatase inhibitors or tamoxifen are increasing the duration of therapy. Already aromatase inhibitors are used for a full 5 years after 5years of tamoxifen [61] and there is an ongoing trial ATLAS testing the effectiveness of long (10 years) against short (5 years) adjuvant tamoxifen treatment of breast cancer. The introduction of extended antihormonal therapy to treat and prevent breast cancer therefore has consequences with the development of antihormonal drug resistance.

Though, the clinical application of the SERM concept has proven itself to be successful, drug resistance remains an important issue arising from long-term SERM treatment. Studies have shown that after long-term SERM treatment, the pharmacology of the SERMs changes from an inhibitory antioestrogenic state to a stimulatory oestrogen-like response [62].

Antihormone Drug Resistance

Clinical and laboratory studies have identified three possible mechanism for the antihormone drug resistance to tamoxifen: the patient can influence the metabolism of tamoxifen, the ER-positive tumour can be intrinsically resistant or the ER positive tumour can initially respond and subsequently develop acquired tamoxifen resistance.

Activation of tamoxifen occurs when it is metabolized via demethylation to N-desmethyltamoxifen and subsequently gets transformed to the hydroxy metabolite endoxifen [63]. Endoxifen is formed by the CYP2D6 enzyme system [64], but there are genetic variants in the population that can influence drug metabolism. It is estimated that mutant alleles of the wild-type CYP2D6 enzyme variants are present in 10% of the population, thus meaning that these patients should be considered for an antioestrogenic therapy, other than tamoxifen i.e. aromatase inhibitors if they are postmenopausal. Side effects, that arise during treatment with tamoxifen, influence compliance and efficacy. An important side effect of tamoxifen is hot flashes and many patients become non compliant and stop therapy or use selective serotonin reuptake inhibitors (SSRIs) to reduce hot flashes. But it appears that hot flashes are good as tamoxifen must be metabolized into the potent antioestrogen endoxifen by the CYP2D6 enzyme. Unfortunately, SSRIs (fluoxetine and paroxetine) are also potent inhibitors of the CYP2D6 enzyme [65]. Therefore, symptom treatment can potentially undermine the efficacy of treatment with tamoxifen if the incorrect SSRI is employed. Venlafaxine is the recommended SSRI as there is a low affinity for the CYP2D6 enzyme system.

Forty percent of ER-positive metastatic breast cancers are intrinsically resistant to tamoxifen treatment. These tumours are identified as ER-positive and PR-negative tumours and only 40% [66] respond to antihormonal therapy. In contrast, ER/PR-positive tumours have an 80% response rate to endocrine therapy. In early studies it was noted that PR induction by oestrogen is impaired, through the epidermal growth factor receptor 1 (HER-1; EGFR) pathway [67], and that paracrine growth factor stimulation undermines the effectiveness of antioestrogen therapy [68]. These observations were expanded using breast cancer cells transfected with insulin-like growth factor receptor and by the examination of large tumour databases [69],[70]. Insulin-like growth factor also reduces PR synthesis, so a general mechanism emerged that growth factor pathways impair ER signal transduction to initiate PR induction. Intrinsic tamoxifen resistance occurs in HER-2/neu-, PR negative, ER positive breast cancer cells that also have increased levels of SRC-3 coactivator [71]. Though, this patient category is only 10 to 15%, it provides a clue about who to test to avoid tamoxifen treatment. A retrospective analysis showed that patients with ER positive, PR-negative tumours would most likely respond better to aromatase inhibitor treatment than to tamoxifen [72], however, these data have subsequently not been confirmed [72],[73].

Finally, and most intriguingly long-term tamoxifen treatment can induce acquired resistance in breast cancers that are ER/PR-positive. Acquired resistance to tamoxifen is unique as the tumours are SERM stimulated for growth [74]. The first laboratory model [62],[40],[41] of transplantable tamoxifen resistant cells demonstrated that 1) tamoxifen or oestrogen can cause tumours to grow, 2) tumours require a liganded receptor to grow, 3) an aromatase inhibitor (oestrogen deprivation) or a pure antioestrogen that causes ER destruction would be useful second line agents, 4) there was cross resistance with other SERMs [75].

However, it is the study of acquired antihormone resistance that has not only allowed the development of appropriate second line treatment strategies for patients (aromatase inhibitors or fulvestrant), but also has advanced our understanding of the apoptotic biology of high dose oestrogen as an effective therapy for breast cancer in the 1940's [7].

Evolution of Antihormone Drug Resistance

An obstacle to the progress in therapeutics is a clear understanding of the changes that occur in the breast cancer cell, as a consequence of exhaustive antihormonal therapies. It is presumed that the cancer cell must create a sophisticated survival network and suppress the natural process of apoptosis to subvert the continuous inhibitory signal through the ER. Currently, numerous model systems exist to study antihormone resistance. Some are engineered to enhance the likelihood of resistance [71] and others are engineered by transfection of the aromatase gene to study resistance to aromatase inhibitors and compare them with tamoxifen [76]. In contrast, others have chosen to develop models naturally through selective pressure either *in vivo* or *in vitro*. The natural selection approach is to either continuously transplant the resulting SERM resistant breast cancer into SERM-treated athymic animals [77,78] or to employ strategies *in vitro* that use continuous SERM treatment [79-81] or long term oestrogen deprivation in culture [82,83].

In order to better understand the biological consequences of extended antioestrogen treatment on the survival of breast cancer, we have elucidated distinct phases of resistance with the use of unique models of tamoxifen-resistant breast cancer developed *in vivo* (Fig.(**3**). The model for the treatment phase of breast cancer was developed by injecting ERα-positive MCF-7 cells into athymic mice and supplementing them with post-menopausal doses of oestradiol (E2) (86–93 pg/ml) [84]**.** These MCF-7 tumours were oestradiol (E2)-stimulated and tamoxifen (TAM)-inhibited. Phase I TAM-resistant breast tumours developed with short term treatment (<2 years) with tamoxifen and were stimulated to grow by both E2 and tamoxifen [62,85]. The novel model of Phase II resistance to tamoxifen was observed when breast tumours were treated long-term with tamoxifen for more than 5 years (MCF-7TAMLT). These MCF-7TAMLT tumours were stimulated to grow with tamoxifen but paradoxically inhibited by E2 [77,86,87] (Fig.(**3**). Phase III resistance developed when all known therapies failed and only E2-inhibited growth [88]. However, during the progression from the treatment phase to Phase III resistance, a cyclic phenomenon was observed where initially E2-inhibited growth of Phase II TAM-resistant tumours followed by re-sensitization to E2 as a growth stimulant [86]. These E2 re-stimulated MCF-7 tumours from Phase II tamoxifen-resistant tumours were growth inhibited by no treatment, TAM, and fulvestrant demonstrating complete reversal of drug resistance to tamoxifen. In addition to tamoxifen-resistant tumours, oestradiol, at physiologic concentrations, has also been shown to induce apoptosis in long term oestrogen deprived (LTED) breast cancer cells *in vitro* and *in vivo*. It should be noted that in the past, pharmacologic oestrogen was a routinely employed therapy that resulted in durable responses with regression of disease [7]. Oestrogen therapy has yielded as high as 40% response rate as first-line treatment in patients with hormonally sensitive breast cancer with metastatic disease [89] and approximately 31% in patients heavily pre-treated with previous endocrine therapies [90]. What is still unclear, however, is the mechanism of oestradiol-induced apoptosis in breast cancer cells that are stimulated by tamoxifen or that grow spontenously when deprived of oestradiol for a long time (> 1 year).

Mechanisms of Oestrogen Induced Apoptosis

Apoptosis is a form of programmed cell death that is executed by a family of proteases called caspases, which can be activated either by cell-surface death receptors (i.e., the extrinsic pathway) or by perturbation of the mitochondrial membrane (i.e., the intrinsic pathway) [91] (Fig.(**4**). Components of the extrinsic pathway include the death receptors FasR/FasL, DR4/DR5, and tumour necrosis factor (TNFR), whereas the intrinsic pathway centers on the mitochondria, which contain key apoptogenic factors such as cytochrome *c* and apoptosis inducing factor (AIF) [91] (Fig.(**4**). In the intrinsic pathway, the integrity of mitochondrial membranes is controlled primarily by a balance between the antagonistic actions of the pro-apoptotic and anti-apoptotic members of the Bcl-2 family. Bcl-2-family proteins comprise three principal subfamilies: (1) anti-apoptotic members, including Bcl-2/Bcl-x_L, which possess the Bcl-2 homology (BH) domains BH1, BH2, BH3, and BH4; (2) pro-apoptotic members, such as Bax, Bak, and Bok, which have the BH1, BH2, and BH3 domains; and (3) BH3-only proteins, such as Bid, Bim, Bad, Bik, and Puma, which generally possess only the BH3 domain [92]. The Bcl-2 family of proteins regulate apoptosis by altering mitochondrial membrane permeabilization and controlling the release of cytochrome *c*.

Mechanistic studies have used either SERM-stimulated models [80,87] or long-term oestrogen deprived MCF-7 breast cancer cell lines [82,83,93] to demonstrate a link between oestradiol-induced apoptosis and activation of the FasR/FasL death-signaling pathway. Osipo and coworkers [87,93] reported that physiologic levels of oestradiol induced regression of tamoxifen-stimulated breast cancer tumours by inducing the death receptor Fas and suppressing the antiapoptotic/prosurvival factors NF-κB and HER2/neu. A similar finding was reported by Liu and coworkers [80] for raloxifene (Ral)-resistant tumours. These investigators reported that the growth of Ral-resistant MCF-7/Ral cells *in vitro* and *in vivo* was repressed by oestradiol by a mechanism involving increased Fas expression and decreased NF-κB activity. Furthermore, Song and coworkers [82] showed that MCF-7 cells deprived of oestrogen for up to 24 months (MCF-7LTED) *in vitro* expressed high levels of Fas compared to the parental MCF-7 cells, which do not express Fas and treatment of the MCF-7/LTED cells with oestradiol resulted in a marked increase in Fas ligand (FasL) in these cells. Apart from the death receptor pathway, there is also evidence that the mitochondrial pathway is involved in oestradiol induced apoptosis. Oestradiol induced apoptosis occurs in a LTED breast cancer cell line named MCF-7:5C by activating the Bcl-2 family proteins (Fig.(**4**). In MCF-7:5C cells the expression of several pro-apoptotic proteins—including Bax, Bak, Bim, Noxa, Puma, and p53—are markedly increased with oestradiol treatment and blockade of Bax and Bim expression using siRNAs almost completely reversed the apoptotic effect of oestradiol. Oestradiol treatment also led to a loss of mitochondrial potential and a dramatic increase in the release of cytochrome c from the mitochondria, which resulted in activation of caspases and cleavage of PARP. Furthermore, overexpression of anti-apoptotic Bcl-x_L was able to protect MCF-7:5C cells from oestradiol-induced apoptosis. This particular study was one of the first to show a link between oestradiol-induced cell death and activation of the mitochondrial apoptotic pathway using a breast cancer cell model resistant to oestrogen withdrawal. It is worth noting that Song and coworkers [94] have also demonstrated the importance of Bcl-2 in mediating oestradiol-induced apoptosis in LTED cells. These investigators reported that basal bcl-2 level was markedly elevated in LTED cells and that knockdown of bcl-2 expression with siRNA dramatically sensitized these cells to the apoptotic action of oestradiol. At present, there is renewed interest in developing small molecule inhibitors of bcl-2 as anticancer cell and antiangiogenic agents [95].

Apart from its action on the mitochondria, there is evidence that Bcl-2 also possesses antioxidant property. Bcl-2 overexpression increases cellular glutathione (GSH) level which is associated with increased resistance to chemotherapy-induced apoptosis [96]. GSH is a water-soluble tripeptide composed of glutamine, cysteine, and glycine. It is the most abundant intracellular small molecule thiol present in mammalian cells and it serves as a potent intracellular antioxidant protecting cells from toxins such free radicals [97,98]. Changes in GSH homeostasis have been implicated in the etiology and progression of a variety of human diseases, including breast cancer [99] and studies have shown that elevated levels of GSH prevent apoptotic cell death whereas depletion of GSH facilitates apoptosis [100]. Recently, our laboratory has found evidence which suggests that glutathione participates in retarding apoptosis in antihormone-resistant MCF-7:2A human breast cancer cells and that depletion of this molecule by L-buthionine sulfoximine (BSO), a potent inhibitor of glutathione biosynthesis, sensitizes these resistant cells to oestradiol-induced apoptosis [101]. GSH levels were elevated ~60% in antihormone-resistant MCF-7:2A cells compared to wild-type MCF-7 cells and unlike MCF-7:5C cells, the MCF-7:2A cells failed to undergo apoptosis following 1 week of treatment with physiological concentrations of oestradiol. In the presence of BSO (100 μM), however, 1 nM oestradiol caused a dramatic increase in apoptosis which was observed as early as 48 hours with maximum induction observed at day 7. It is worth noting that the concentration of BSO (100 μM) used in this study is clinically achievable [102]. Furthermore, early phase clinical trials of BSO at doses resulting in both peripheral and tumour GSH depletion show that BSO can be safely administered to patients with refractory disease [103]. Thus it is possible that future clinical studies of BSO infusions combined with low dose oestrogen hold the promise of improving disease control for patients with antihormone resistant ER positive metastatic breast cancer.

Clinical Exploitation of Oestrogen-Induced Apoptosis

Laboratory studies uniformly demonstrate that low concentrations of oestrogen can cause apoptotic tumour cell death following profound oestrogen deprivation with antihormones. This can be viewed as an enhanced vulnerability to oestrogen when Phase II antihormone resistance is developed consistent with the earlier use of high dose oestrogen to treat breast cancer in women 2-3 decades after menopause [7]. The question that needs now to be answered is how can this new laboratory knowledge be translated into patient care?

Several clinical trial groups [104] are currently addressing this issue. In our own case, we are recruiting patients with metastatic breast cancer who have succeeded and experienced treatment failure with at least two successive endocrine therapies and we are determining the efficacy of a 12-week purge of high-dose oestradiol (30 mg daily) therapy (Fig.(**5**). The goal is to confirm and extend the previous study published by Lonning and colleagues [90] and then to determine the minimum dose of oestradiol necessary to induce the anticipated 30% response rate [90]. Based on our previous laboratory studies [86] we propose to re-treat responding patients with the aromatase inhibitor anastrozole to determine efficacy. Overall, our clinical program is part of a multi-institutional Center of Excellence grant BCO50277 entitled “A New Therapeutic Paradigm for Breast Cancer Exploiting Low-Dose Oestrogen-Induced Apoptosis” that will map the survival and death pathways of our models and integrate clinical material to determine the validity of the laboratory-derived molecular mechanisms and, ultimately, to address the issue of why the majority of tumours do not respond to oestrogen alone. We reason that knowledge of the new apoptotic biology of oestrogen could be enhanced in the future in much the same way as the modest responses of tamoxifen and raloxifene were enhanced to benefit patients. The philosophy is to deploy the right treatment at the right time, for the right patient.

Perspectives

Our proposed model clinical trial now provides opportunities to test compounds in associations with oestrogen as an apoptotic trigger. Pre-clinical data from our laboratory [101] clearly show that it is possible to enhance the apoptotic effect of low dose oestradiol by combining it with BSO. We propose that the combination of BSO and oestradiol could be used to improve the efficacy of oestradiol as an apoptotic agent if glutathione depletion is fundamental to tumour cell survival. Phase I clinical trials of BSO at doses resulting in both peripheral and tumour GSH depletion show that BSO can be safely administered to patients with refractory disease. BSO was administered intravenously twice daily either alone or together with chemotherapy to cancer patients whose disease had progressed despite multiple lines of previous chemotherapy [103,105].

We propose that inhibitors of survival pathways will enhance the apoptotic/growth inhibitory effects of oestrogen. Bcl-2 (B-cell lymphoma/leukemia-2) is a low molecular weight protein that is localized to the mitochondria and endoplasmic reticulum that acts as a key inhibitor of apoptosis. Expression of Bcl-2 is essential for growth of certain tumour cell lines *in vitro* and has been found to be upregulated in a variety of tumour types *in vivo* [106,107]. It is widely believed that some cancers evade apoptosis and obtain a survival advantage through aberrant expression of Bcl-2. To date, several independent groups have developed small-molecule inhibitors of Bcl-2 as antitumour agents [95]. These inhibitors encompass various drugs that bind the antiapoptotic Bcl-2 family members with more or less efficacy. Oblimersen (Genasense; G3139; Genta Inc, Berkeley Heights, NJ) is an anti-Bcl-2 antisense oligonucleotide which has reached phase III clinical trials in combination therapy [108]. Peptide-based drugs have also been shown to attenuate Bcl-2 activity [109] and to activate Bax [110]. There are also natural inhibitors of Bcl-2 which include tea polyphenols such as catechins and theaflavins [111,112] and the natural polyphenol derivative gossypol. Inspired by the potential of natural Bcl-2 inhibitors, several research groups have developed specific inhibitors of Bcl-2. HA14-1 was the first Bcl-2 binding ligand to be discovered using computer-based screening strategies using the predicted structure of Bcl-2 [113]. Other small-molecule inhibitors of Bcl-2 include TW-37 [113,114] and ABT-737 [115], both of which have better efficacy than HA14-1 [116]. Overall, the small-molecule inhibitors of Bcl-2, although they are not magic bullets, have great therapeutic potential and are proving to be an important investigative tool for understanding the function of Bcl-2.

There is strong clinical evidence that trastuzumab, a monoclonal antibody targeting the human epidermal growth factor receptor (HER) two tyrosine kinase receptor, is an important component of first-line treatment of patients with HER-2 positive metastatic breast cancer [117-120]. In particular, the combination with taxanes and vinorelbine has been established [121]. In the preoperative setting inclusion of trastuzumab has significantly increased the pathological complete response rate. Results from large phase III trials evaluating adjuvant therapy in HER-2 positive early breast cancer indicate that the addition of trastuzumab to chemotherapy improves disease-free and overall survival [122]. Based on our preclinical studies [80,87], we have found that HER-2 is an important target of oestradiol-induced apoptosis, hence, the possibility exist that the combination of oestradiol therapy with that of trastuzumab might have beneficial effects. In addition to trastuzumab, there is also pertuzumab (2C4, Omnitarg®) (Genentech Inc. San Francisco, CA, USA), a monoclonal antibody directed against HER-2 that sterically blocks dimerization of HER-2 with HER-1 and HER-3 [123,124]. It is currently under early clinical evaluation, phase I data have shown that the drug is well tolerated and clinically active [125]. Ertumaxomab (Rexomun®) (Fresenius Biotech GmbH, Munich, Germany) is a novel trifunctional, bispecific antibody that targets HER-2 and CD3. A phase I study among 17 patients with HER-2 positive metastatic breast cancer has demonstrated strong immunologic responses with this antibody [126]. In addition, recent studies have reported that ertumaxomab induces cellular cytotoxicity against various tumour cell lines including cells with low expression of HER-2 [127]. Thus, this antibody may provide a new therapeutic option for breast cancer patients with low expression of HER-2.

Apart from monoclonal antibodies, the use of tyrosine kinase inhibitors to target HER-2 has also shown great promise. Lapatinib (Tyverb®, GW572016) (GlaxoSmithKline, Middlesex, UK) is a dual tyrosine kinase inhibitor of both HER-1 and HER-2, and of Akt and mitogen activated protein kinase (MAPK). Preclinical studies have demonstrated that this compound inhibits growth and induces apoptosis in breast cancer cell lines [128]. Results from phase I/II trials suggest that the compound has activity against several tumour types, in particularly breast cancer [129,130]. In addition, xenograph studies have shown that lapatinib may be able to restore tamoxifen sensitivity [131]. The compound has also been evaluated in combination with aromatase inhibitors in preclinical and clinical studies [132,133]. However, increased ER signaling has been demonstrated in biopsies from HER-2 positive breast tumours treated with lapatinib. This finding might indicate that ER signaling could be involved in lapatinib-resistance [134]. A phase I study of lapatinib in combination with the aromatase inhibitor letrozole in patients with solid tumours showed a positive response [132]. A phase III study comparing letrozole with letrozole plus lapatinib in patients with ER/PR-positive metastatic breast cancer has recently completed enrolment [135]. Data have not yet been published.

Conclusion

The discovery of a new biology of oestradiol-induced apoptosis provides a unique signal transduction pathway to exploit in the treatment of metastatic breast cancer that has become refractory to exhaustive antihormone therapy. The clinical clues with the use of high-dose oestrogen therapy [7,9,90] have now been supported by a wealth of laboratory data defining apoptotic mechanisms. It is plausible to consider that the methodical evaluation of monoclonal antibodies and small molecule tyrosine kinase inhibitors to prevent breast cancer survival could amplify the apoptotic actions of oestradiol in a select group of patients. Indeed, if a study of the molecular biology of oestrogen-induced apoptosis can precisely define the mechanism then the molecules involved will become the target for a new drug group. These new drugs may be able to precipitate apoptosis in ER-negative breast tumours or indeed be used universally to treat cancers other than breast cancer. All will depend on tissue selectivity.

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Figure Legends

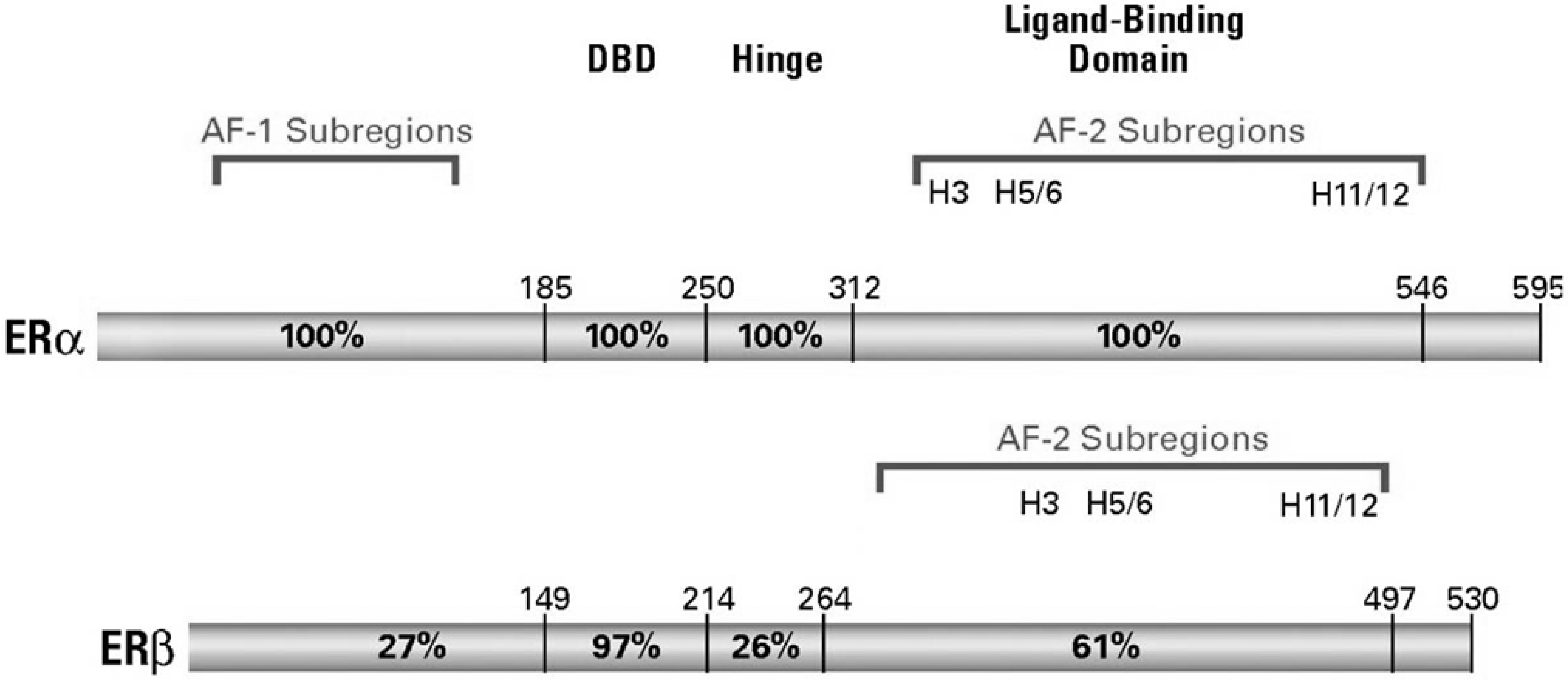
Fig.(**1**). ERα and ERβ functional domains. ERα and ERβ proteins both are members of nuclear hormone receptor superfamily and bind oestrogens and regulate transcription of oestrogen responsive genes. They share the highest degree of amino acid sequence, 61 and 97% in LBD and DBD respectively, however A/B and D domains have only 27% and 26% amino acid homology respectively, however with lack of homology of A/B domain between ERα and ERβ, functional studies have indicated that ERβ lacks AF-1 activity.

Fig.(**2**). Coregulators and Oestrogen receptor action. Structurally different ligands change the conformation of the oestrogen receptor and thus program them to bind coactivators with different enzymatic activities or either co-repressors. Coactivators are necessary for the receptor to bind to the promotor regions of the oestrogen responsive genes and then be degraded by 26S proteasome complex sibsequently, which is necessary for sustaining the gene transcription. In case of antioestrogen binding, coactivators prevent activation of oestrogen responsive gene transcription.

Fig.(**3**). Evolution of antihormone resistance. Acquired resistance of breast cancer cells occurs during long-term treatment with SERMs and is evidenced by SERM-stimulated growth of these cells. Tumours still exploit oestrogen for growth when the SERM treatment is stopped, meaning that a dual-transduction pathway has developed. At this point aromatase inhibitors are effective as they destroy the ER. This phase of drug resistance is called phase I. Continued exposure to SERMs eventually leads to oestrogen-independent autonomous growth of breast cancer cells, continuing to be SERM-stimulated. However, these cells now respond by apoptosis to reintroduction of oestradiol, rather than growth. This phase of drug resistance is called phase II.

Fig.(**4**). The proposed subcellular mechanism for oestrogen induced apoptosis of SERM resistant (Phase II) and long term oestrogen deprived (aromatase inhibitor-resistant) breast cancer. In SERM-resistant breast cancer, there is an induction of the Fas receptor/Fas ligand resulting in activation of caspase 8 and induction of apoptosis (programmed cell death). In long term oestrogen deprived breast cancer, the oestradiol ER complex alters Bcl-2 family protein expression which then activates the mitochondria causing cytochrome *c* release, caspase 9 activation, and PARP cleavage, ultimately resulting in cell death.

Fig.(**5**)**.** Clinical protocol to investigate the efficacy of oestradiol treatment to induce apoptosis in long-term endocrine refractory breast cancer. An anticipated treatment plan for third-line endocrine therapy. Patients must have responded and experience treatment failure with two successive antihormone therapies to be eligible for a course of low-dose oestradiol therapy for 3 months. The anticipated response rate is 30% and responding patients will be treated with anastrozole until relapse. The overall goal is to increase response rates and maintain patients for longer on antihormone strategies before chemotherapy is required.


DBD
Hinge
Ligand-Binding Domain
AF-1 Subregions
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H3
H5/6
H11/12
185
250
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97%
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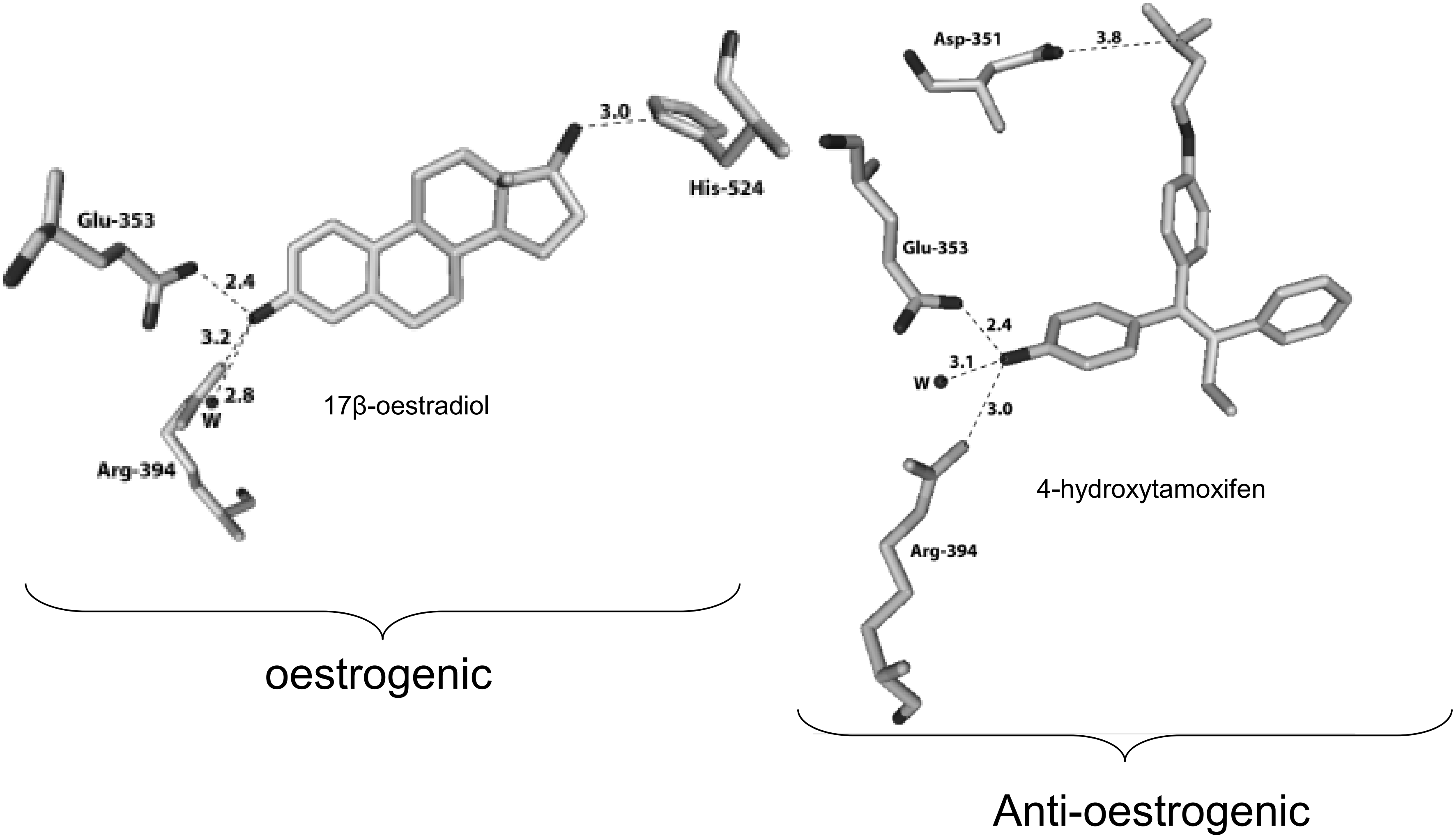
61%

Figure 1


Glu-353
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3.2
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Arg-394
17β-oestradiol
3.0
His-524
oestrogenic
Asp-351
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Glu-353
2.4
3.1
W
3.0
Arg-394
4-hydroxytamoxifen
Anti-oestrogenic

Figure 2A

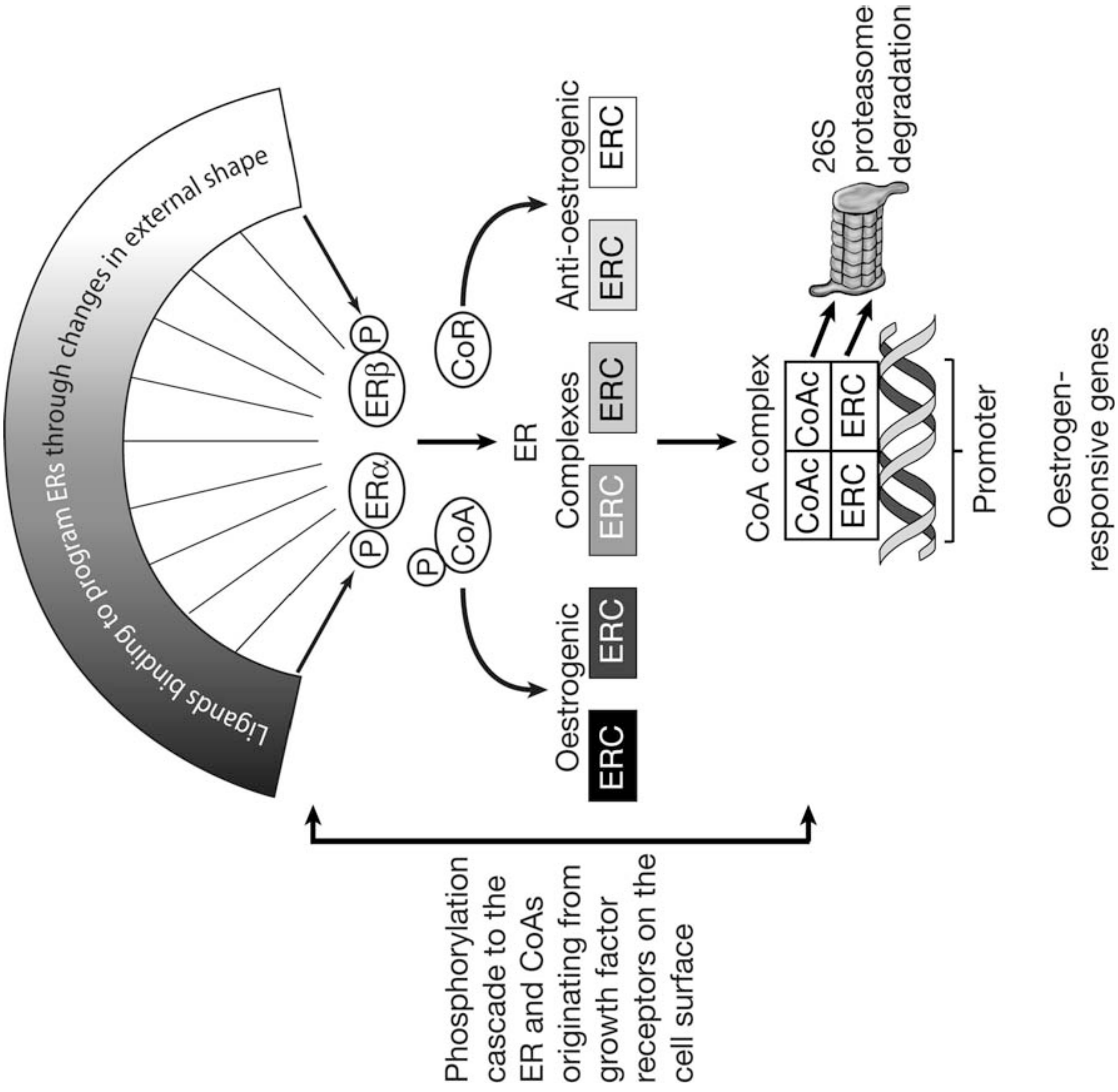

Ligands binding to program ERs through changes in external shape
P
ERα
ERβ
P
P
CoA
CoR
ER
Complexes
Oestrogenic
Anti-oestrogenic
ERC
ERC
ERC
ERC
ERC
ERC
CoA complex
CoAc
CoAc
ERC
ERC
26S
proteasome
degradation
Promoter
Oestrogen-
responsive genes
Phosphorylation
cascade to the
ER and CoAs
originating from
growth factor
receptors on the
cell surface

Figure 2B

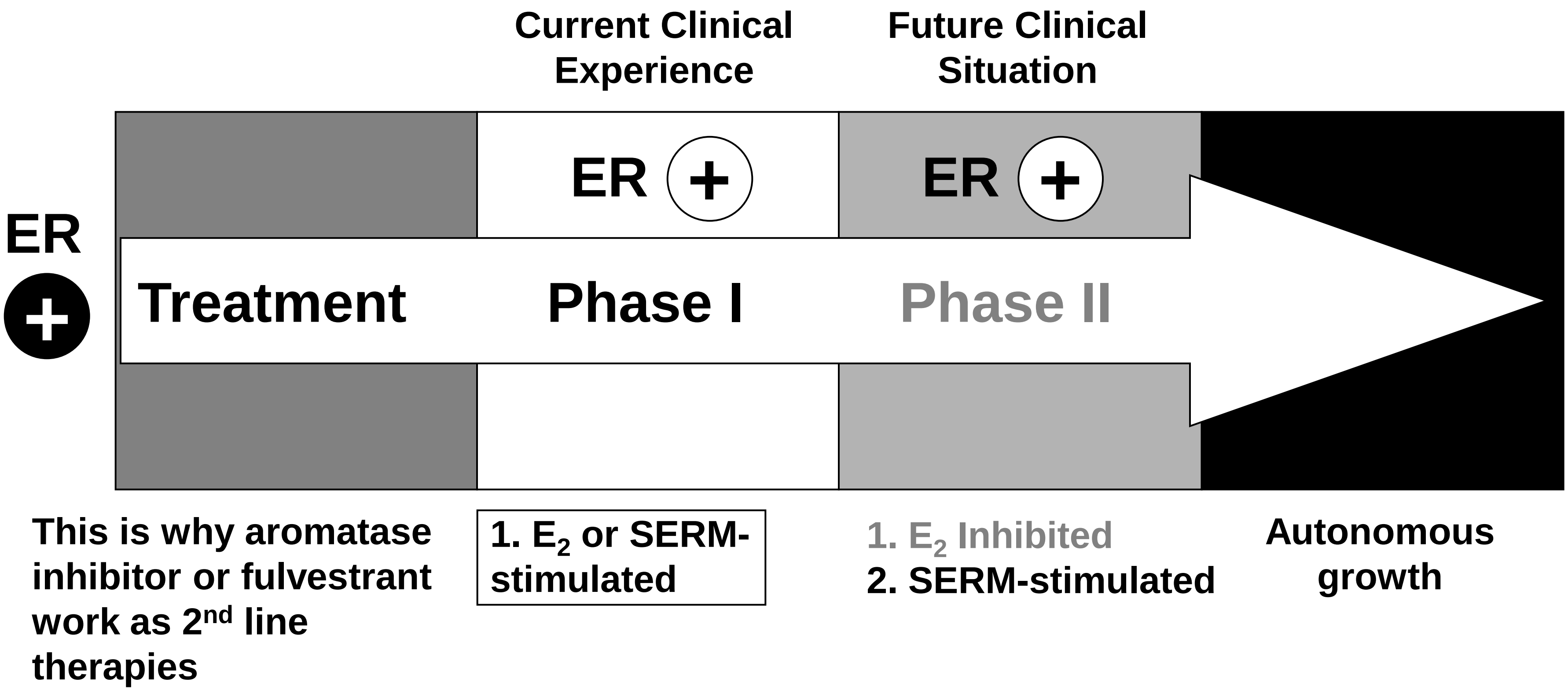

Current Clinical Experience
Future Clinical Situation
ER +
ER +
ER +
Treatment
Phase I
Phase II
This is why aromatase inhibitor or fulvestrant work as 2nd line therapies
1. E_2 or SERM-stimulated
1. E_2 Inhibited
2. SERM-stimulated
Autonomous growth

Figure 3

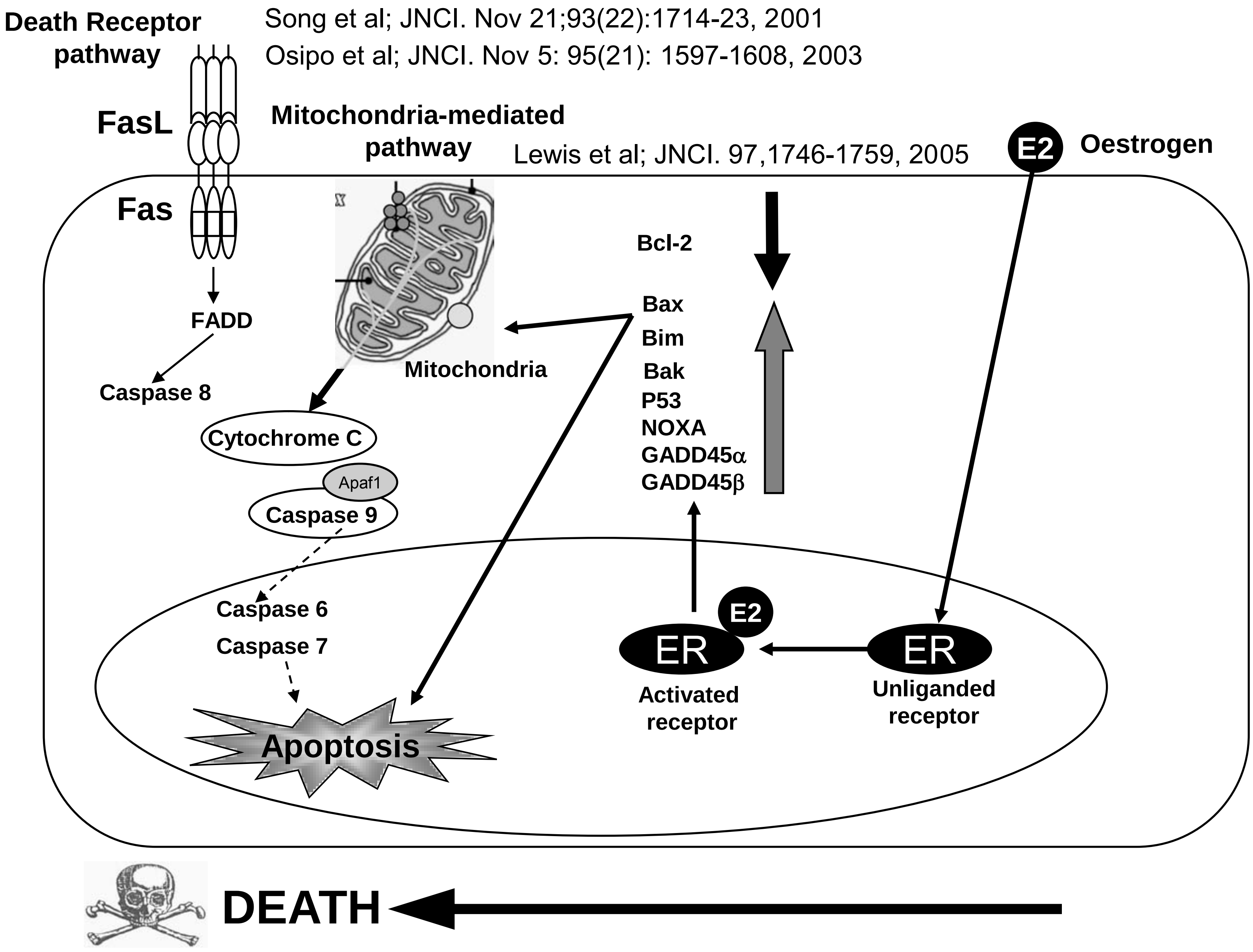
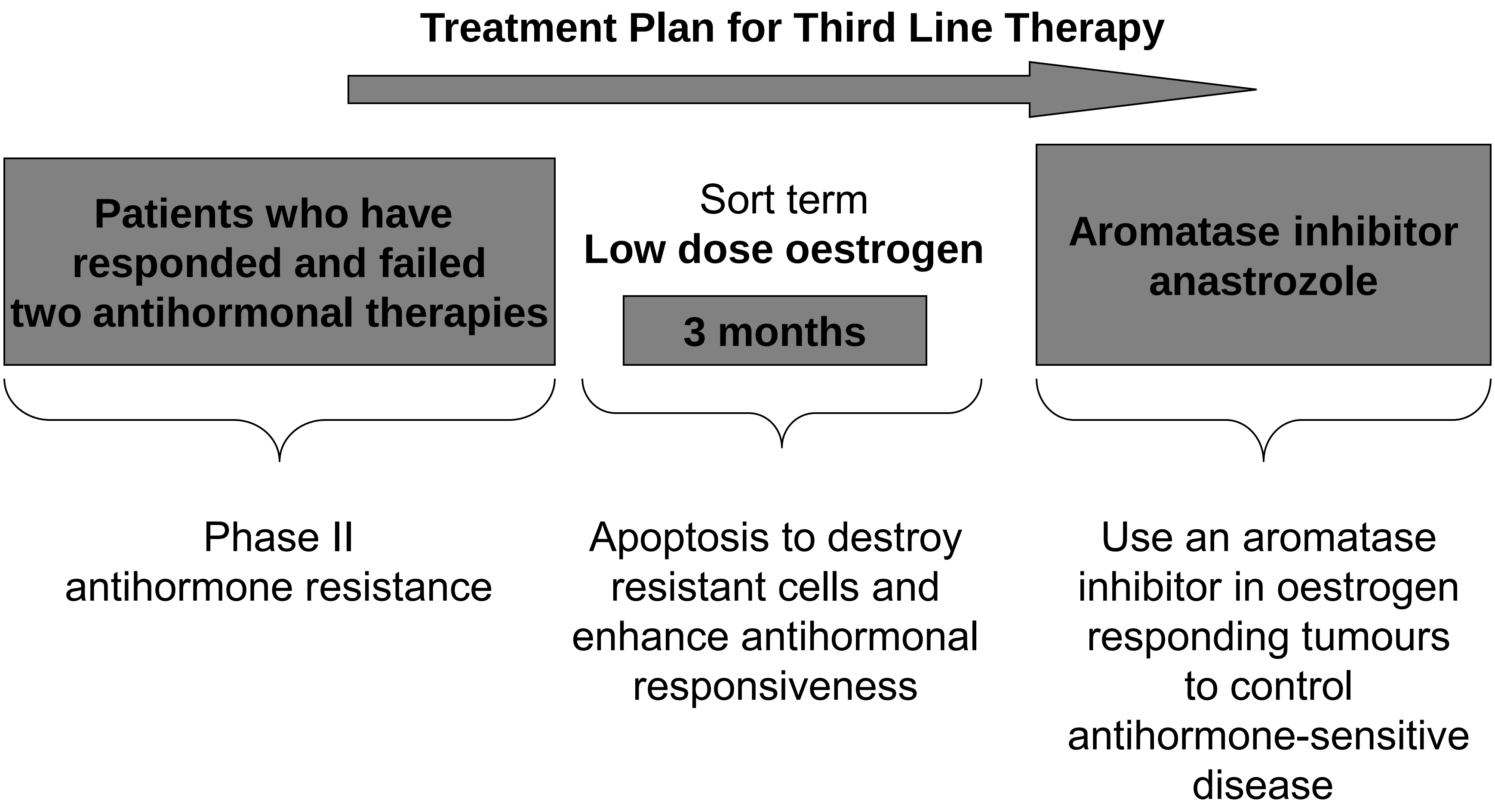

Death Receptor pathway
Song et al; JNCI. Nov 21;93(22):1714-23, 2001
Osipo et al; JNCI. Nov 5: 95(21): 1597-1608, 2003
FasL
Mitochondria-mediated pathway
Lewis et al; JNCI. 97,1746-1759, 2005
E2
Oestrogen
Fas
Bcl-2
Bax
Bim
Bak
P53
NOXA
GADD45α
GADD45β
FADD
Caspase 8
Mitochondria
Cytochrome C
Apaf1
Caspase 9
Caspase 6
Caspase 7
Apoptosis
E2
ER
Activated receptor
ER
Unliganded receptor
DEATH

Figure 4

Figure 5



Treatment Plan for Third Line Therapy

Patients who have responded and failed two antihormonal therapies

Phase II
antihormone resistance

Sort term
Low dose oestrogen

3 months

Apoptosis to destroy resistant cells and enhance antihormonal responsiveness

Aromatase inhibitor anastrozole

Use an aromatase inhibitor in oestrogen responding tumours to control antihormone-sensitive disease

Potential of Selective Estrogen Receptor Modulators as Treatments and Preventives of Breast Cancer

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ABSTRACT

Estrogen plays vital roles in human health and diseases. Estrogen mediates its actions almost entirely by binding to estrogen receptors (ER), alpha and beta which further function as transcription factors. Selective estrogen receptor modulators (SERMs) are synthetic molecules which bind to ER and can modulate its transcriptional capabilities in different ways in diverse estrogen target tissues. Tamoxifen, the prototypical SERM, is extensively used for targeted therapy of ER positive breast cancers and is also approved as the first chemo-preventive agent for lowering breast cancer incidence in high risk women. The therapeutic and preventive efficacy of tamoxifen was initially proven by series of experiments in the laboratory which laid the foundation of its clinical use. Unfortunately, use of tamoxifen is associated with de-novo and acquired resistance and some undesirable side effects. The molecular study of the resistance provides an opportunity to precisely understand the mechanism of SERM action which may further help in designing new and improved SERMs. Recent clinical studies reveal that another SERM, raloxifene, which is primarily used to treat post-menopausal osteoporosis, is as efficient as tamoxifen in preventing breast cancers with fewer side effects. Overall, these findings open a new horizon for SERMs as a class of drug which not only can be used for therapeutic and preventive purposes of breast cancers but also for various other diseases and disorders. Major efforts are therefore directed to make new SERMs with a better therapeutic profile and fewer side effects.

Key Words: breast cancer, osteoporosis, estrogen receptor, tamoxifen, raloxifene, SERMs, endocrine therapy, drug resistance

1. INTRODUCTION

Breast cancer incidences and death rates have dropped significantly during recent years, which is associated strongly with improvement in early detection methods and decrease of menopausal hormone replacement therapy (HRT) [1, 2]. HRT, in the form of estrogen alone or estrogen plus progesterone, had been widely used since the 1960s until recent years, to treat conditions associated with aging as well as unpleasant menopausal symptoms. HRT was also known to protect post-menopausal women from osteoporosis and also thought to protect women from heart disease and Alzheimer's disease. However, the Women's Health Initiative (WHI) study indicated that taking estrogen with or without progesterone for 5 or more years placed the women at higher risk of breast cancer, Alzheimer's disease, heart disease, blood clot and stroke, although HRT is effective to reduce the risks of osteoporosis and colon cancer [3, 4]. The Million Women Study (MWS) conducted in the UK also showed that women taking HRT were more likely to develop breast cancer [5], endometrial cancer [6] and ovarian caner [7]. In the US, the use of estrogen-plus-progestin HRT has dropped almost 50% when the WHI announced their findings in 2002, and this was followed by a sharp 7% decrease of new breast cancers in 2003 [2]. Although the decrease of HRT uses is not the sole reason leading to less breast cancer incidences, much effort has been focused on finding more effective and safer compounds to replace HRT which not only relieve menopausal symptoms but also prevent and treat hormone-responsive cancers. One most promising approach is to use selective estrogen receptor modulators (SERMs).

SERMs are synthetic compounds that bind to estrogen receptors alpha and/or beta (ERα and/or ERβ) and exert estrogenic or antiestrogenic activities in a tissue/cell-specific manner. The first SERM that has been used successfully in the clinic to prevent and treat breast cancer is tamoxifen, a failed postcoital contraceptive that evolved into the "gold standard" for breast cancer treatment [8, 9]. Tamoxifen is estimated to have saved the lives of over 400,000 women with breast cancer [8]. The second generation SERM, raloxifene (formally called keoxifene), failed as a treatment for breast cancer but is effective against osteoporosis and prevents breast cancer at the same time. Raloxifene is as effective as tamoxifen to reduce invasive breast cancer risks without an increase in the risk of endometrial cancer observed with tamoxifen [10]. Indeed a recent study suggests that raloxifene might even be effective in preventing endometrial cancer [11]. These findings have acted as a catalyst for the search of new SERMs which are estrogen-like in bones and circulating lipids but antiestrogenic in women's reproductive organs and therefore are anti-cancer agents. However, there are problems associated with the current SERMs such as drug resistance and side effects. For example, both tamoxifen and raloxifene increase both hot flashes and blood clots [12].

Besides SERMs, other endocrine therapies target the ER indirectly to prevent and treat breast cancer. Aromatase inhibitors (AIs) that block the synthesis of estrogen from androgen in peripheral tissues have been extensively studied and show efficacy equivalent or superior to tamoxifen to treat postmenopausal breast cancer [13]. Since the mechanism for AIs to treat ER-positive breast cancer is to deplete estrogen in postmenopausal patients, they do not increase risks of endometrium cancer or blood clot and may be a better choice for postmenopausal breast cancer patients than tamoxifen. However, AIs are not effective in premenopausal women with actively functioning ovaries because AIs do not inhibit ovarian estrogen production. In addition, AIs lack the estrogenic protective function for cardiovascular diseases or osteoporosis. As a result, the side effects of AIs are mostly consistent with estrogen deprivation. AIs are associated with a greater incidence of bone loss and musculoskeletal symptoms, and probably higher risk of cardiovascular disease suggested by adjuvant trials comparing AIs and tamoxifen [14]. However, AIs are associated with a lower incidence of gynecological symptoms, thromboembolic diseases and hot flashes compared to tamoxifen in adjuvant setting [14]. Another strategy is to use selective estrogen receptor down-regulators (SERDs), such as fulvestrant, that cause degradation of ERs. Fulvestrant has been approved to treat advanced breast cancer after tamoxifen failure, and a recent phase III trial indicated that fulvestrant and AI exemestane were equally effective with a similar safety profile [15].

Resistance is a common problem associated with endocrine therapy therefore alternative treatment strategies without cross-resistance are necessary. Compared to the pure anti-estrogenic actions like AIs or fulvestrant, an ideal SERM with beneficial estrogenic effects has great potential for breast cancer prevention and treatment, especially in postmenopausal women as they often suffer from unpleasant symptoms resulting from lower estrogen. A perfect SERM would reduce the risk of breast cancer, ovarian cancer and uterine cancer, as well as strengthen the bone, prevent coronary heart disease, strokes and Alzheimer's disease, and relieve menopausal discomfort like hot flashes and vaginal atrophy [12].

The complicated outcome of SERMs action cannot just be explained by turning on or off the ERs and their downstream genes. Although much new knowledge is being developed, we are still evolving in our understanding of the detailed mechanism of SERMs and their interaction with the ERs. In the past decade, another group of protein factors, nuclear receptor coregulators, have been identified that are essential for modulating the functions of SERMs and ERs. In this article, we will review the evolving understanding of the molecular mechanisms of SERMs action in the context of other signal transduction pathways and nuclear receptor coregulators, as well as the problems associated with the application of SERMs as a treatment or preventative for breast cancer. Finally, the new SERMs with potential as new agents to treat or prevent breast cancer will be described.

2. MECHANISM OF ESTROGEN ACTION

2.1. Structure and Function of ER

The existence of estrogen binding protein was first predicted by Elwood Jensen and colleagues in early 1960's [16]. The first ER cDNA, now known as ERα, was later cloned in the mid-1980's [17, 18]. In 1996, an additional ER was cloned from rat prostate [19] and designated as ERβ. The action of estrogen in cells is therefore almost entirely mediated by these two related but distinct subtype of estrogen receptors, ERα and ERβ. Both receptors function as ligand activated transcription factors which can bind the cognate DNA sequences known as estrogen responsive elements (ERE), and activate transcription. The ER proteins can be structurally subdivided into six domains on the basis of the functions controlled by the region, as shown in Fig. (**1**). The A/B domain contains one of the two transcriptional activation functions (AFs), designated as AF1 which is involved in estrogen-independent activation of transcription. Another activation function domain, AF2, is located in the E domain which also harbors the ligand binding domain (LBD), and is involved in estrogen/ ligand dependent activation [20, 21]. The

ERβ has 97% homology in the DBD and 61% homology in the LBD with ERα suggesting differential ligand binding capability of ERs [21].

SERMs, the molecules which can bind to ERα and/or β and can either stimulate estrogen-like actions (agonist) or oppose estrogen actions (antagonist) in various estrogen target tissues and cells. This pharmacologic knowledge advanced studies to decipher the details of the molecular mechanism of estrogen action in different cell and tissue types.

The structural studies of a SERM complexed with the LBD of ERα and ERβ reveal that re-orientation of the AF2 helix (helix 12) after the binding of the SERM to the hydrophobic pocket of the LBD [22, 23]. The interaction of amino acid Asp351 of ERα with the alkylaminoethoxyphenyl side chain of tamoxifen or raloxifene is crucial to prevent the recruitment of coactivators to the SERM-receptor complex surface [22, 23]. Using different mutants of ERα for the amino acid Asp351, it was shown that shielding and neutralization of Asp351 by the side chain of raloxifene is critical in defining the antiestrogenicity of this SERM. Furthermore, it has been shown that changing the Asp351 from aspartate to glycine (D351G) abolishes the estrogen-agonist activity of the tamoxifen-ER complex, while retaining its antagonistic property. The AF2 region of the agonist-bound receptor is particularly important for the interactions of steroid receptor coactivators (SRCs 1-3) via the interacting amino acid motif LxxLL, known as nuclear receptor interacting domain (NRID). It is important to note that the affinity of ERs for these NRIDs of SRCs is highly dependent upon the ER subtype, α and β, and ligand bound to the ER [24-26]. Recruitment of these co-activator(s) is also responsible for facilitating the activation of estrogen responsive genes by modifying the chromatin structure and activating the transcriptional machinery. Additionally, SERMs may also show differential AF1 activity mediated by co-repressor binding. Using ERE-reporter constructs, it has been shown that the AF1 domain of ERα is actively involved in agonist-induced gene expression whereas the AF1 domain of ERβ is involved very weakly [27].

Estrogen can also modulate the expression of genes by another mechanism in which the receptor complex can interact with other transcription factors such as activating protein 1 (AP1) or stimulating protein 1 (Sp1) through a process known as a tethering mechanism. Intriguing differences are observed in the mechanism of action between ERα and ERβ through an AP1 site. In the presence of estrogen, ERα induces AP1 driven reporter activity but ERβ has no effect [28]. The raloxifene bound ERβ complex can induce transcriptional activity through the AP1 site but the activity through ERα bound to raloxifene is negligible.

ERs also act in a non-genomic manner initiated from the cell membrane. These actions are very fast (seconds to minutes) and occur without RNA or protein synthesis. They often mobilize second messenger molecules such as Ca^{2+} and cAMP, and are associated with protein kinase cascades such as PI3K/Akt and MAPK [29, 30]. Several explanations have been offered to explain these effects. There could be a subpopulation of nuclear ERs associate with the plasma membrane, either through posttranslational modification such as palmitoylation or mediated by scaffold proteins such as caveolin-1 and MNAR, since ERs do not have a transmembrane domain [29, 30]. Another membrane bound protein, G protein coupled receptor GPR30, was identified in recent years that mediates non-genomic actions of estrogen [31, 32]. The cellular localization of GPR30 is still controversial. Some evidence suggests it is at plasma membrane [33, 34] and other evidence suggests it is in the endoplasmic reticulum [32]. GPR30 binds to 17β-estradiol, tamoxifen and fulvestrant with high affinity [33] and is associated with breast cancer metastasis and transactivation of the epidermal growth factor receptor (EGFR) [35].

2.2. Co-Regulators

The co-regulators are protein molecules which can physically interact with the liganded or un-liganded ERs and modulate the transcription of the genes. The transcriptional activation or repression of the responsive genes is a combinatorial function of ligand-receptor interaction, recognition of cognate DNA sequence and recruitment of specific co-regulators onto the promoter of the gene. The assembly of the whole transcriptional complex is also dependent upon the affinity of the above mentioned individual components among themselves and their relative concentrations in the cell. Co-regulators play defining roles in the final tissue outcome in terms of transcriptional activation or repression mediated by estrogen or SERMs. The co-regulators can be broadly classified on the basis of their function, as co-activators which promote the activation of the transcriptional process, or co-repressors which are associated with repression of transcription of genes (Fig. (**2**)).

2.2.1 Co-Activators

Presently, around 200 co-activators are known, which are associated with 48 nuclear receptors [36]. The family of p160 proteins known as steroid receptor co-activators (SRCs) have been studied extensively. The relative abundance of SRC1 in uterine cells is responsible for the agonistic activity of tamoxifen, whereas in breast cancer cells, with low SRC1 levels, tamoxifen acts as an estrogen antagonist [37]. However, raloxifene, another related SERM, does not recruit SRC-1 even in the uterine cells [37], suggesting that the interaction with specific ligand which elicits a unique conformation of the receptor is critical for the interaction of co-regulators. These observations further provide an explanation for the earlier studies, where tamoxifen have been reported to induce growth of endometrial cancer cells but not of breast cancer cells in athymic mice [38] and also that estrogen agonistic properties of raloxifene is less in endometrial cancer cells [39]. These finding also translate very well to clinical experience [40]. In addition, the SERMs can enhance the stability of the co-activators (SRC1 and SRC3) and thereby influence the transcriptional capability of other nuclear receptors [41]. Post-translational modifications of the co-activators, including but not limited to phosphorylation, methylation, ubiquitylation, sumoylation and acetylation, can also regulate the gene activation by influencing the ability of the co-activators to interact with ER and other components of the transcriptional complex [34-36]. The understanding of structure-function relationship of ligands at the ER has formed the basis of designing effective new SERMs with fewer side effects.

2.2.2. Co-Repressors

Co-repressors are functional counterparts of co-activators, which are associated with transcriptionally inactive promoters and help repress the expression of genes [42]. Fewer co-repressors have been reported compared to the co-activators. In the case of ER, the co-repressors are known to interact with the un-liganded and/or antagonist bound receptor. The two most extensively studied co-repressors in connection with ER are Nuclear receptor corepressor (NCoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT). The ER bound to raloxifene or 4-hydroxytamoxifen (a potent antagonist metabolite of tamoxifen) is known to recruit NCoR and SMRT to the promoters of estrogen responsive genes and repress transcription [43-45]. It has been shown that inhibition of NCoR or SMRT with monoclonal antibodies can enhance the agonistic property of 4-hydroxytamoxifen [46]. Moreover, using fibroblasts from NCoR null mice, 4-hydroxytamoxifen was shown to be a relatively potent ERα agonist [47]. The critical role of NCoR and SMRT in 4-hydroxytamoxifen-induced arrest of cell proliferation of ERα positive breast cancer cells is confirmed because 4-hydroxytamoxifen-stimulated cell cycle progression now occurs in NCoR-and-SMRT-deficient breast cancer cells [48]. However not all estrogen responsive genes are activated by 4-hydroxytamoxifen in NCoR and SMRT deficient cells, clearly indicating that additional molecules are important in SERM-induced repression of estrogen responsive genes. Indeed, there are several other co-repressor proteins known for ER. Metastasis associated protein 1 (MTA1) is a corepressor found to mediate the ER transcriptional repression [49]. Another corepressor, known as repressor of estrogen action (REA) potentiates the inhibitory effects of anti-estrogens including 4-hydroxytamoxifen. Additionally, REA interacts with ER and competes with the co-activator SRC1 for binding to the estrogen bound ER [50, 51]. This again emphasizes the fact that the relative levels of co-regulators may be important in deciding the outcome of the SERM action. The proteasomal regulation of NCoR is another factor which may influence the SERM action. Degradation of NCoR occurs through the 26S proteasome, which is mediated by seven in absentia homologue 2 (Siah2) [52]. Interestingly, estrogen mediated upregulation of Siah2 in ER positive breast cancer cells has been implicated in the proteasomal degradation of NCoR, and subsequent de-repression of NCoR regulated genes [53].

In addition to acting as a "transcriptional adapter" between the receptors and the transcriptional machinery, the coregulator itself or its complex possess various enzymatic activities such as acetylation, phosphorylation, methylation or de-acetylation by which they are able to modify the local chromatin structure thereby making the local environment conducive for gene expression or repression. Intrinsic histone acetyl transferase activity was found to be associated with co-activator SRC1 which helps in the activation of transcriptional expression [54]. In contrast, the 4-hydroxytamoxifen bound ER complex which recruits the co-repressors NCoR and SMRT is associated with histone de-acetylases and other chromatin modifying enzymes [37, 55]. The deacetylase activity promotes transcriptional repression [37, 55]. Interestingly, another enzyme in the co-activator complex, CARM1 (coactivator associated arginine methyltransferase 1) has recently been implicated in modifying the coactivator itself and inducing the degradation of the complex [56]. This suggests the ability of the enzymes in the complex to modify other proteins in its own complex apart from a role in the modification of chromatin.

With this background of the molecular biology of SERM action, it is now appropriate to describe our evolving understanding about drug resistance. This is important not only because tumor drug resistance is the consequence of long term SERM administration, but also because new knowledge will aid patients with the development of novel treatment strategies for SERM-resistant breast cancer.

3. DRUG RESISTANCE TO SERMs

There are three types of resistance to SERMs based on the mechanism: metabolic resistance, intrinsic resistance and acquired resistance [57].

3.1. Metabolic Resistance

Metabolic resistance to tamoxifen is mostly related to CYP2D6, an enzyme product that metabolizes tamoxifen into its active forms 4-hydroxytamoxifen and endoxifen [58]. This has been extensively reviewed recently and will only be briefly mentioned here [13, 59]. CYP2D6 is genetically polymorphic and 5-8% of Caucasian subjects are CYP2D6 "poor metabolizers" thus are less likely to benefit from tamoxifen treatment, although it has been shown that these women tolerate tamoxifen better and tend to remain on the drug for longer [59]. The genotype of CYP2D6 has been shown in multiple clinical trials to be directly related to the outcome of tamoxifen use, however, the results are not always consistent. Eight studies indicated that CYP2D6 "poor metabolizer" genotypes have worse outcome of breast cancer patients who received tamoxifen but two studies contradicted this conclusion [60]. In addition to the genotype of CYP2D6, it is important to consider that other drugs may interact with the enzyme system and block the metabolic activation of tamoxifen. Unfortunately, selective serotonin reuptake inhibitors (SSRIs) that are used to relieve the menopausal side effects of tamoxifen are also metabolized by CYP2D6 and block the metabolic activation of tamoxifen. The proper choice of SSRI is therefore important so as not to impair tamoxifen metabolism. The SSRI of choice is venlafaxine that has only a low affinity for the CYP2D6 enzyme [61]. Although these emerging data about CYP2D6 genotypes and the drug interaction between tamoxifen and SSRIs are important, it is perhaps too early to use CYP2D6 status to routinely choose between tamoxifen and aromatase inhibitors to treat postmenopausal women with breast cancer. At present, an international consortium is evaluating the overall CYP2D6 status of completed clinical trials with tamoxifen to assemble a large scale retrospective analysis of the worth of genotyping. The aim is to answer the question of whether "poor metabolizers" should avoid tamoxifen use.

3.2. Intrinsic Resistance

Approximately 30% ER-positive breast cancer patients do not respond to tamoxifen [62]. This type of resistance is referred to as "de novo" resistance or intrinsic resistance. Clinical studies showed that only 40% patients with ER-positive, progesterone receptor (PR)-negative breast cancers are responsive to anti-estrogen treatment (tamoxifen or endocrine ablation) compared to 80% responsive rate in ER-and-PR-positive patients [58, 59]. Historically, the status of PR has been regarded as an indicator of a functional ER pathway, since expression of PR is regulated by estrogen. On the other hand, recent evidence suggested that the absence of PR is associated with excessive growth factor signaling such as overexpression of HER2 [63, 64], which has been known to impair estrogen induction of PR and reduce the effectiveness of tamoxifen treatment for breast cancer [65]. However, the negative association between PR and HER2 seems more evident in older women (> 45 yrs) [66] and it remains controversial that PR-status could be used for clinical decision on choosing between tamoxifen or AIs [67].

Growth factor signaling, especially through epidermal growth factor (EGF) pathway, has been studied extensively in the past two decades and linked to SERM resistance. This has been recently reviewed [68] and will only be briefly summarized here. EGF binds to ErbB family of cell surface receptors that include four closely related receptor tyrosine kinases: EGFR (ErbB-1), HER2/c-neu (ErbB-2), HER3 (ErbB-3) and HER4 (ErbB-4). Overexpression of HER2 has been clinically linked to less response to endocrine therapies and worse prognosis [69-71], so has the overexpression of EGFR [72]. Different ErbB family members can form heterodimers and activate multiple signalling pathways including PI3K/Akt and MAPK. The major molecular mechanisms leading to SERM resistance can be summarized as follows: 1. Activation of downstream kinase cascade results in the phosphorylation of ER at key residues (Ser106/107, 118, 167, 305 and Thr 311) which activates transcription in a ligand-independent manner. Phosphorylation may change the binding of ER with ligands, DNA and coregulators, which may ultimately alter the activity of SERMs [73]. For example, phosphorylation of ER at Ser167 by Akt and Ser118 by the MAPK pathway both cause ligand-independent activation [74-76]. A recent study showed that phosphorylation of ER at Ser305 altered the orientation between the C-terminus of ER and SRC-1 that led to the recruitment of ER transcription coactivators and RNA polymerase II even in the presence of tamoxifen [77]. 2. Phosphorylation of ER co-regulators is equally important as the phosphorylation of ER itself, since phosphorylated co-activators have increased activity in the presence of SERMs [78-80]. Phosphorylation of co-repressors such as SMRT is associated with the co-repressor's nuclear export and impaired transcriptional suppressing function [81]. 3. Other than enhancing the transcriptional activity of the ER by phosphorylation, overexpression of EGFR or HER2 increases the non-genomic actions of ER, and SERMs may now act as estrogen agonists via the membrane effects of ER [82, 83]. In addition to the EGF signal pathway, the insulin-like growth factor (IGF) signal pathway is also involved in tamoxifen resistance [84]. It can activate PI3K/Akt pathway [71] and turn on genes that are otherwise activated by estrogen [85, 86].

Dysregulation of ER co-regulators is another major contributor to intrinsic SERM resistance. Overexpression of both AIB1 (SRC-3, ACTR, p/CIP, RAC3, TRAM-1) and HER2 have been shown to convert tamoxifen into an estrogen agonist in breast cancer cells [79]. Elevated AIB1 was found to associate with tamoxifen resistance, DNA-nondiploidy, high S-phase fraction and HER2 amplification in samples from clinical study [87]. Although a study indicated that high expression of AIB1 was not associated with relapse during tamoxifen treatment [88], AIB1 was shown to associate with tamoxifen resistance in breast cancers that overexpressed ErbB family proteins [88, 89]. AIB1 might be a predictor marker for tamoxifen ineffectiveness in ER-positive, HER2-positive and PR-negative breast cancer. On the other hand, low expression of ER co-repressor NcoR is associated with shorter relapse-free survival in breast cancer patients who only received tamoxifen after surgery [90]. Based on the emerging importance of co-regulators and tamoxifen resistance, one novel approach to overcome tamoxifen resistance is by the use of disulfide benzamide (DIBA) to disrupt the zinc finger in the ERα DNA binding domain. The approach facilitates ERα dissociation from coactivator AIB1 and concomitant association of corepressor NcoR without changing the phosphorylation of HER2, MAPK, Akt or AIB1 [91].

Another group of regulators associated with tamoxifen resistance are microRNAs (miRNA). These are naturally occurring single-stranded RNAs with the length of 21-23 nucleotides that do not code for proteins. They regulate gene expression mainly by inducing target mRNA degradation or inhibiting translation (protein synthesis). Dysregulation of miRNAs is associated with many cancers including breast cancer [92, 93]. Two recent studies show that miRNA-221/222 are upregulated in tamoxifen-resistant breast cancer cells and primary tumors, and they may contribute to tamoxifen resistance by down-regulating p27Kip1 or ERα [94, 95].

3.3. Acquired Resistance

Breast cancer patients who initially respond to tamoxifen later develop “acquired resistance” that is characterized by tamoxifen stimulated growth. This can be replicated in the laboratory with MCF-7 xenograft tumors implanted in ovariectomized athymic mice. Tamoxifen initially inhibits estrogen stimulated tumor growth but eventually some tumors start to grow during tamoxifen therapy [96]. These tumors now grow in response to either estrogen or tamoxifen and stop growing with no treatment or during treatment with fulvestrant [96]. The laboratory model is consistent with the clinic observation that aromatase inhibitor or fulvestrant are equally effective after the failure of tamoxifen treatment [97, 98]. It therefore appears that ER remains fully functional in the laboratory model of acquired tamoxifen resistance. In clinical studies, only 17-28% patients with acquired tamoxifen resistance have a loss of ER function [99, 100], and it is more likely that acquired resistance is associated with the stimulation of other growth/survival pathways [101]. For example, activated mammalian target of rapamycin (mTOR, downstream of PI3K/Akt and MAPK pathway) and c-Src (downstream of EGFR/HER2) were observed in breast cancer cells and mTOR and c-Src inhibitors can restore tamoxifen sensitivity in these cells, respectively [102, 103]. Several genes involved in cell proliferation and survival have altered expression level in breast cancer cells with acquired tamoxifen resistance. Examples of genes which down regulation is associated with acquired tamoxifen resistance include cyclin-dependent kinase inhibitors p21Cip [104] and p27Kip [105]. Examples of genes which upregulation is associated with tamoxifen acquired resistance include cyclin-dependent kinase 10 (CDK10) [106] and anti-apoptotic protein survivin [107].

Laboratory observation showed that acquired tamoxifen-resistant breast cancer cells/tumors respond differently to estrogen, and three phases of tamoxifen-resistance have been described, which seems to depend on the length of tamoxifen exposure [12]. Tumors with phase I resistance are stimulated by estrogen and tamoxifen but inhibited by AIs and fulvestrant; tumors with phase II resistance are stimulated by tamoxifen but are inhibited by estrogen due to apoptosis; tumors with phase III resistance (automatous growth) grow in a hormone-independent manner that is not responsive to either AIs or fulvestrant or SERMs, but estrogen still exerts apoptotic actions on those tumors [12]. The laboratory models suggest a new treatment strategy, in which limited duration, low-dose estrogen can be used to purge phase II- or phase III-resistant breast cancer cells so that the tumors will be responsive to antiestrogen therapy again. Phase II clinical study is ongoing to test this treatment plan [108].

Most studies on SERM-resistance are related to tamoxifen and little is known about raloxifene resistance. Based on a few studies on raloxifene resistance using cell culture and animal models, raloxifene-resistant tumors are likely to have similar properties as tamoxifen-resistant ones [109]. Raloxifene-resistant MCF7 cells generated by long-term exposure to raloxifene *in vitro* are also resistant to tamoxifen *in vitro and in vivo*. They exhibit phase II SERM-resistance as estradiol treatment causes tumor regression by inducing G2/M cell cycle arrest and apoptosis [110]. Another raloxifene-resistant breast tumor model generated by exposing MCF7 breast tumors to raloxifene *in vivo* exhibits phase I SERM-resistance whose growth is stimulated by tamoxifen, raloxifene and estrogen [109]. Interestingly, protein levels of EGFR and HER2 are also increased in this phase I raloxifene-resistant tumor model, which suggests raloxifene-resistant tumors share similar molecular mechanisms as tamoxifen-resistant ones [109].

Overall, the classifications of different forms of antihormonal drug resistance can be used as a basis to evaluate the pharmacology of new SERMs. The goal is to improve on tamoxifen, the pioneer that over the past 30 years found ubiquitous long term applications in the treatment and prevention of breast cancer.

4. NEW SERMs

The discovery of the first antibiotic penicillin initiated a search for further antibiotics to delay drug resistance and to target specific diseases. Similarly, the successful clinical application of tamoxifen in medicine has resulted in the investigation of numerous related molecules to develop the “ideal SERM”. However, it has been challenging to find a SERM that is superior to tamoxifen, which retains or extends its benefit to treat and prevent breast cancer but with fewer side effects. Tamoxifen maintains bone density in animals [111] and humans [112] so SERMs are being developed to treat osteoporosis, but the potential to prevent breast cancer and uterine cancer will also increase their clinical value and commercial success. The core structures of SERMs are diverse, including triphenylethylene, benzothiophene, chromene (benzopyran), naphthalene, indole and steroid, but each of the newer SERM is really a mimic of tamoxifen, raloxifene or estradiol. The development of dozens of SERMs have been discontinued due to ineffectiveness for human disease or severe side effects, but several new SERMs are under active investigations with great potential in breast cancer treatment and/or prevention, alone or in combination with other type of drugs. In addition, since the identification of ERβ in 1996 [19], ER subtype selective SERMs have been developed which could potentially be used as breast cancer preventives. Thus this area of medicinal chemistry remains an important topic of interest as new ER regulated targets emerge. We will review the current status of several agents that are either approved or in the process of drug development (summarized in Table).

4.1. Tamaoxifen-like SERMs

4.1.1. Toremifene (Fareston)

Toremifene (**2**) is a chlorinated tamoxifen analogue which has been approved in the US and several other countries for the treatment of metastatic breast cancer. Its structure is shown in Fig. (**3**). Toremifene is as effective as tamoxifen in the treatment of ER-positive breast cancer but with the potential of fewer genotoxic effects, since it does not produce DNA adducts in rat liver and human endometrium [113]. The mechanism for the reduced genotoxicity of toremifene can be explained as follows: Tamoxifen-DNA adducts are primarily formed via sulfonation of the α-hydroxylated tamoxifen metabolites, but the α-hydroxy metabolites of toremifene is poorly esterified or sulfonated, and even sulfonated α-hydroxy toremifene, α-sulfoxytoremifene, reacts poorly with DNA [114, 115]. However, there are some reports to show toremifene induces DNA damages and hepatocarcinogenesis in rats [116, 117].

The effects of toremifene and tamoxifen on bones are similar [118], as are the endometrial effects. However, a recent safety evaluation demonstrates that secondary endometrial cancer incidence is lower with toremifene than with tamoxifen and is similar to that with raloxifene [119]. Nevertheless, toremifene stimulates the growth of human endometrial cancer implanted in athymic mice in the same way as tamoxifen [120]. The positive effects of toremifene on lipid profiles are superior to tamoxifen's. Toremifene lowers the low-density lipoprotein (LDL) cholesterol to a level similar to that seen with tamoxifen, but unlike tamoxifen, toremifene slightly increases high-density lipoprotein (HDL) cholesterol and lowers triglycerides in the serum [121, 122].

Cross-resistance with tamoxifen is an important issue to consider when using toremifene for recurrent breast cancer because the majority of patients have received or failed adjuvant tamoxifen. Toremifene is completely cross-resistant with tamoxifen in human breast tumors implanted in athymic mice [123], as well as in breast cancer patients [124, 125]. Therefore, toremifene would not be effective as a second-line endocrine therapy after tamoxifen failure and may offer no therapeutic advantages over tamoxifen as an adjuvant therapy.

In recent years, toremifene has been developed to treat other estrogen-related diseases. Toremifene is effective to treat mastalgia in some small phase II trials [126, 127], and is also effective at decreasing prostate cancer incidences in a high-risk population [128]. In addition, a recent multicenter randomized phase III trial showed that toremifene increased bone density and improved lipid profile in men receiving androgen deprivation therapy for prostate cancer [129, 130].

4.1.2. Ospemifene (Deaminohydroxytoremifene, FC-1271a)

Ospemifene (**3**), or deaminohydroxytoremifene, is a metabolite of toremifene (Fig. (**3**)). Like toremifene, ospemifene is generally well tolerated and has a favorable safety profile. It does not induce DNA adducts in mice [131], rats [132] and monkey [133]. Ospemifene exerts a very weak estrogenic effect on endometrial histology, like raloxifene and decreases cholesterol [134]. However, unlike tamoxifen or raloxifene, ospemifene has significant estrogenic effects on vaginal epithelium [134-136] and is being developed for postmenopausal vaginal atrophy, a chronic condition experienced by about 40% postmenopausal women. Ospemifene is being evaluated in a phase III trial that has already recruited 826 women. Early results suggested that a 12-week course of ospemifene treatment significantly relieves symptoms of dryness in the vagina.

Ospemifene has showed promise in the prevention and treatment of osteoporosis. Cell culture studies indicated that ospemifene inhibits osteoclast formation and bone resorption and protects osteoblast-derived cells from apoptosis [137, 138]. In a recent phase II trial to compare effects of ospemifene and raloxifene on biochemical markers of bone turnover in postmenopausal women, ospemifene showed similar effects as raloxifene in regulating most of the bone markers examined, and at the 90-mg dose, ospemifene increased procollagen type I N propeptide (PINP) more than raloxifene [139]. Ospemifene is currently in phase III development for the treatment of postmenopausal osteoporosis.

Studies based on animal models suggest ospemifene might be effective in breast cancer prevention. Ospemifene prevented dimethylbenzanthracene (DMBA)-induced mammary tumors in female Sencar mice as effectively as tamoxifen, while raloxifene was not effective [140]. In a transplantable mouse model of ductal carcinoma in situ (DCIS), ospemifene had inhibitory effects equivalent to tamoxifen in terms of tumor growth and progression [141]. Nevertheless, the chemoprevention effects of ospemifene in breast cancer need to be further studied and substantiated by clinical trials.

4.1.3. GW5638 (DPC974) and GW7604

GW 5638 (**4**) is a derivative of tamoxifen with an acrylate side chain in place of the dimethylaminoethoxy side chain in tamoxifen. GW7604 (**5**) is the 4-hydroxy version of GW5638, analogous to the major metabolite of tamoxifen, 4-hydroxytamoxifen. Their structures are shown in Fig. (**3**). GW5638 functions as a full ER agonist in bone and the cardiovascular system but as an antagonist in breast and endometrial system in rodent models [142].

Although the structures of tamoxifen and GW5638/GW7604 are similar, GW5638/GW7604 acts with a mechanism different from tamoxifen/4-hydroxy tamoxifen as suggested by the following evidence: 1. GW7604 acts as an antagonist in MDA-MB-231 cells transfected with wild-type ERα, but 4-hydroxytamoxifen acts as an agonist [143]; 2. Phage display experiments indicate that GW7604 bound ERα or ERβ is associated with different peptides from 4-hydroxytamoxifen, raloxifene or fulvestrant bound ERs [144]; 3. GW5638 inhibits the growth of tamoxifen-resistant breast tumor xenograft [144, 145]; 4. The crystal structure of the ERα LBD bound by GW5638 shows that GW5638 induces a distinct conformation of H12 in the ERα AF2 region, which increased exposure of hydrophobic residues and results in ERα destabilization in MCF7 cells [146].

GW5638 and GW7604 are also classified as selective estrogen receptor down-regulators (SERDs) because they induce ERα degradation, a property observed with the pure antiestrogen fulvestrant which was approved for the treatment of metastatic breast cancer [147]. However, a recent report [148] indicates that GW5638 induces ERα degradation through a different mechanism from fulvestrant and another SERD RU58,668, as the protein/protein interaction surface on ER required for fulvestrant-induced degradation is not necessary for GW5638-induced degradation. The fact that GW5638 has a unique mechanism to antagonize estrogen function and induces ER degradation in breast cancer cells makes it a possible second line therapy after tamoxifen failure and as an alternative to fulvestrant. Currently, GW5638 is under clinical development under the name DPC974 [148].

4.2. Raloxifene-like SERMs

4.2.1. Arzoxifene (LY353381)

Arzoxifene (**7**) is a derivative of raloxifene with the ketone group replaced by an ether group and the hydroxy group is replaced by a methoxy group (Fig. (**4**)). These modifications have improved the pharmokinetic properties [149]. Arzoxifene has antiestrogenic effects on breast and endometrium but pro-estrogenic effects on bone and lipids [150]. Arzoxifene is cross-resistant in some but not all tamoxifen-stimulated breast tumor xenografts [151]. Phase II clinical trials indicate that arzoxifene is effective to treat tamoxifen-sensitive or tamoxifen-refractory patients with advanced or metastatic breast cancer [152] and patients with recurrent or advanced endometrial cancer [153] with minimus toxicity. However, a phase III trial showed arzoxifene was inferior to tamoxifen to treat patients with locally advanced and metastatic breast cancer [154]. The main role of arzoxifene may reside in its chemoprevention potential since it is more potent than raloxifene in pre-clinical studies [149].

The breast cancer chemoprevention property of arzoxifene has been studied with animal models and small short-term clinical trials. Arzoxifene effectively prevented nitrosomethylurea (NMU)-induced mammary tumor in rats [140] and induced apoptosis of breast cancer cells in rodent models especially when used in combination with rexinoid LG100268, a selective ligand for the retinoid X receptors (RXR) [155]. In two phase I clinical trails of women with newly diagnosed ductal carcinoma in situ or T1/T2 invasive cancer, arzoxifene did not demonstrate a significant reduction of tumor cell proliferation compared to placebo in 2-6 weeks treatment [156]. However, there were some favorable findings, such as a decrease of serum insulin like growth factor I (IGF-1) vs IGF binding protein 3 (IGFBP3) ratio and an increase of sex hormone binding globulin [156]. Another interesting aspect of the pharmacology of arzoxifene is that it might have chemopreventive properties for ER-negative breast cancer when used in combination with LG100268. A recent study showed that both SERMs arzoxifene and acolbifene alone prevent ER-negative mammary tumor in a mouse model and the effect is synergized with LG100268 [155]. Although the SERMs by themselves are not functional in the treatment of established tumors, together with LG100268 they inhibit proliferation and induce apoptosis in the ER-negative mammary tumors [155]. The mechanism how SERMs prevent tumorigenesis of ER-negative breast tissue is unknown, but the results suggest that arzoxifene has the potential for further clinical development as a chemoprevention drug of both ER-positive and negative breast cancer, especially in combination with rexinoids.

4.2.2 Lasofoxifene (CP-336156, Fablyn)

Lasofoxifene (**8**) has a naphthalene core structure, which is different from all the other SERMs discussed in this article (Fig. (**4**)). However, the crystal structure shows that lasofoxifene fits into the ERα LBD pocket in a similar manner as other ligands [157]. In addition, lasofoxifene-bound ERα LBD has similar conformational features as other SERM-bound ERα LBDs, such as tamoxifen or raloxifene, in which H12 in the "antagonist-bound" conformation and occludes the coactivator binding surface [157]. Lasofoxifene has a high affinity for ER with an IC_{50} of 1.5 nM, which is comparable to 17β-estradiol and higher than tamoxifen and raloxifene [158]. It preserves bone density and lowers serum cholesterol, and also has chemopreventive and chemotherapeutic effects in rat mammary tumor models without any uterine hypertrophic effects [159]. Lasofoxifene is currently undergoing an extensive clinical evaluation for the prevention and treatment of osteoporosis [159]. One advantage of lasofoxifene over raloxifene is its increased oral bioavailability due to the nonpolar naphthalene structure that makes it a poor substrate for intestinal wall glucuronidation [160]. In addition to its effects on bone, lasofoxifene significantly improves symptoms of vaginal atrophy [161] and a recently completed phase III trial indicated that lasofoxifene decreased vaginal pH and improved the vaginal-cell maturation index in osteoporotic postmenopausal women. These effects may be due to the increased vaginal ERβ and androgen receptor protein levels [162]. Lasofoxifene acts as a chemopreventive and treatment in the NMU-induced rat mammary tumor model. The results are similar to the comparator drug tamoxifen [163]. Phase III trials are currently ongoing to evaluate its ability to prevent breast cancer and cardiovascular diseases in postmenopausal women [164].

4.2.3. Pipendoxifene (ERA-923)

Pipendoxifene (**9**) has an indole core structure (Fig. (**4**)). It was designed by adding an alkylaminoethoxyphenyl side chain to zindoxifene (D-16726), a 2-phenylindol based SERM which failed as a treatment for breast cancer [165]. Pipendoxifene, also named ERA-923, mimics the structure of raloxifene and is devoid of uterotrophic activities in immature rats and ovariectomized mice compared to raloxifene [166]. It inhibits the growth of tamoxifen-sensitive and -resistant tumors in rats and mice [167] and is under phase II clinical development for the treatment of tamoxifen-resistant metastatic breast cancer. In a recent study, a combination of pipendoxifene and temsirolimus, which is a mammalian target of rapamycin (mTOR) inhibitor, synergistically inhibited growth of MCF-7 cells and xenograft models even at suboptimal doses, primarily by causing G1 cell cycle arrest [168]. This suggested that combination of a SERM and an mTOR inhibitor might be of clinic value as breast cancer treatments.

4.2.4. Bazedoxifene (TSE-424, WAY-140424)

Bazedoxifene (**10**) is another indole SERM, designed and synthesized at the same time as pipendoxifene with a slight structural difference, as shown in Fig. (**4**) [166]. This SERM is being actively developed to treat osteoporosis with the potential to prevent breast cancer. Bazodoxifene binds to ERα and ERβ with an affinity lower than raloxifene but is less selective for ERα [169]. It inhibits estrogen-mediated proliferation of breast cancer MCF7 cells and increases bone density with little uterine or vasomotor effects in rat models [169]. A Phase III trial with 497 healthy postmenopausal women showed that 6-month bazedoxifene treatment decreases endometrium thickness and uterine bleeding, suggesting antagonistic effects of bazedoxifene in endometrium [170]. Bazedoxifene is currently under review by the Food and Drug Administration (FDA) for the prevention and treatment of postmenopausal osteoporosis. The completed 3-year phase III trial which enrolled 7,492 postmenopausal women with moderate to severe osteoporosis showed bazedoxifene significantly reduced the incidences of vertebral and non-vertebral fracture compared to placebo, while raloxifene was not effective against non-vertebral fracture [171]. No safety concerns related to breast or endometrium were observed, however, a statistical insignificant increase of venous thromboembolic events was observed with groups treated with either bazedoxifene or raloxifene in the same study [172]. Based on studies using rodent models, combination of bazedoxifene and conjugated estrogens exerted positive vasomotor, lipid, and skeletal responses with minimal uterine stimulation [173]. This suggested that pairing SERMs and estrogen might be effective in the treatment of menopausal symptoms and prevention of osteoporosis. However, further studies are needed to examine the effectiveness of bazedoxifene in breast cancer prevention.

4.2.5. Acolbifene (EM-652, SCH57068) and EM-800 (SCH57050)

Acolbifene (EM-652) (**11**) and its orally active prodrug EM-800 (**12**) have a chromene core structure (Fig. (**4**)). They were initially misclassified as pure antiestrogens and their side chain was depicted by analogy with the pure antiestrogen fulvestrant [174]. However, the structure of acolbifene is actually similar to that of raloxifene, and unlike fulvestrant, the antiestrogenic side chain of acolbifene does not mask the mutant ER amino acid D351Y to produce an estrogenic action [175]. In addition, acolbifene and EM-800 act as antiestrogens in mammary and uterine tissues, but have estrogenic effects to prevent bone loss and have a favored function in the regulation of lipid metabolism by lowering plasma cholesterol and triglyceride in rodent models [176, 177]. Therefore, acolbifene and EM-800 should be classified as SERMs.

Acolbifene has the highest ER-binding affinity among all known compounds [178]. Preclinical studies indicated that acolbifene and EM-800 were more potent than tamoxifen, idoxifene, raloxifene, GW-5638, toremifene and droloxifene to inhibit the growth of breast cancer cell lines MCF-7, ZR-75-1, MCF-7 and T47D as well as ZR-75-1 xenograft in mice models [179, 180]. Interestingly, acolbifene caused disappearance of 65% ZR-75-1 xenograft in ovariectomized nude mice, while other SERMs tested (tamoxifen, toremifene, raloxifene, droloxifene, idoxifene and GW 5638) only decreased the tumor growth rate stimulated by estrone [180]. Acolbifene was evaluated as a second line therapy for tamoxifen-refractory breast cancers, since it was regarded as a pure anti-estrogen. In a small clinical trail involved 43 postmenopausal or ovariectomized women with breast cancer who had received tamoxifen for over a year but relapsed, the objective response to EM-800 was 12% with 1 complete response and 4 partial responses [181]. In a phase III trial to compare acolbifene with the aromatase inhibitor anastrozole in breast cancer patients who did not respond to tamoxifen, acolbifene did not show superior antitumor activity to anastrozole and the study was halted [182]. However, acolbifene and EM-800 may be more suitable as first line therapy and a phase III trial for untreated metastatic breast cancer patients is planned [182].

Recent studies indicate that acolbifene might be used in combination with other drugs. Acolbifene synergizes with rexinoid LG100268 in the prevention and treatment of mice with ER-negative mammary tumor [155]. It also synergizes with dehydroepiandrosterone (DHEA), which is a naturally produced prohormone for androgen and estrogen, in the prevention of dimethylbenzanthracene (DMBA)-induced mammary tumors in the rats [183]. A phase III trials of acolbifene plus DHEA for vaginal atrophy and uterine safety has been planned.

4.2.6. CHF4227

CHF4227 (**13**) is a SERM with a chromene (benzopyran) core structure, as shown in Fig. (**4**). Compared with raloxifene, CHF4227 binds to ERα and ERβ with higher affinity and inhibits the uterotropic action of 17alpha-ethynyl estradiol with more potency [184]. CHF4227 significantly prevents the development of DMBA-induced mammary tumors in rats [184]. It preserves bone mass without affecting uterine weight and decreases serum cholesterol and fat mass in ovariectomized rats [185]. A recent phase I study showed CHF4227 is well-tolerated, as 28 days of treatment has a positive effect on the serum lipid profile and bone markers without any negative effects on the endometrium or the fibrinolytic system. Additionally, CHF4227 does not cause vaginal bleeding or hot flashes [186]. These results suggest that CHF4227 is safe and worthy of further clinical development for osteoporosis and the chemoprevention of breast cancer.

4.2.7. SP500263

SP500263 (**14**) was discovered in a screen to identify ER agonist in bone cells [187]. It has a chromene core structure and binds to both ERα and ERβ with high affinity similar to raloxifene's (Fig. (**4**)) [187]. SP500263 inhibits the growth of breast cancer MCF7 cells and xenografts in nude mice, and does not stimulate uterine weight gain in immature rats or ovariectomized adult rats [188, 189]. SP500263 also blocks osteoclastogenesis in human bone cell model [190]. These preclinical results suggest that SP500263 has potential for the treatment of both breast cancer and osteoporosis. However, clinical value of this drug has yet to be determined.

4.3. Steroidal SERMs

4.3.1. HMR3339

All of the SERMs described to this point are non-steroidal. Recently, steroidal SERMs have been described (Fig. (**5**)). In rats, HMP3339 (**15**) not only increases bone mineral density but also restores the mechanical strength at multiple sites even after ovariectomy, and it affects both cortical and cancellous bones, while raloxifene was effective only at cancellous sites [191]. HMR3339 has entered clinical investigation for the prevention of osteoporosis and cardiovascular diseases. In a series of small phase II trials with healthy postmenopausal women, HMR3339 was found to reduce total cholesterol, LDL cholesterol, C-reactive protein (CRP, a pro-inflammatory cytokine and a cardiovascular disease risk factor), asymmetric dimethylarginine (AMDA, a nitric oxide synthase inhibitor) and homocysteine [192-194]. Elevation of AMDA or homocysteine is linked to a high incidence of cardiovascular disease but raloxifene treatment does not reduce the level of either AMDA or homocysteine [192]. HMR3339 reduces concentrations of procarboxypeptidase U (pro-CpU, an inhibitor of fibrinolysis), antithrombin and fibrinogen to a degree similar to raloxifene and shows beneficial effects on some markers of fibrinolysis [195, 196]. Therefore, HMR3339 has potential to prevent cardiovascular diseases and possibly also osteoporosis. However, whether or not there is potential as a cancer preventive has not been determined.

4.3.2 PSK3471

PSK3471 (**16**) is a newly developed SERM with a structure similar to HMR3339 (Fig. (**5**)). It was reported to prevent gonadectomy-induced bone loss in male and female mice, and antagonize estradiol-stimulated MCF-7 cell proliferation [197].

4.4. ER Subtype Selective SERMs

ER α and ERβ have a different tissue distribution and have overlapping but distinct biological functions [198]. Unlike ERα, ERβ expression is not routinely examined in the clinic and its function in breast cancer remains unclear. ERβ expression is found in both normal and breast cancer specimens but does not correlate with ERα expression [199]. It seems that ERβ functions differently if it is expressed alone or co-expressed with ERα in breast cancers. In ERα-positive breast tumors, ERβ often antagonizes the pro-proliferation actions of ERα [200, 201] and its expression is associated with better response to endocrine therapy and a favorable clinical outcome in most cases [202]. Thus ERβ seems to function as a tumor suppressor. However in ERα-negative breast tumors, several studies indicated that the expression of ERβ correlates with proliferation markers such as Ki67 and cyclin A [202, 203], which suggested that ERβ might stimulate cancer growth. In the latter situation, ERβ could serve as an endocrine therapy target in those patients who would otherwise be regarded as ER-negative and have limited choice but chemotherapy. The presence of ERβ in ERα-negative breast cancers may partly explain why some "ER-negative" patients respond to SERMs. The reason that ERβ functions differently in the absence or presence of ERα might be due to the different activities between the ERα/β heterodimer and ERα or ERβ homodimers.

A new direction to consider is the estrogen related receptor (ERR) [204, 205]. There is emerging evidence that ERRα is critical for the growth of ER-negative breast-cancer MDA-MD-231 xenografts in mice [206], as ERRα appears to be involved in angiogenesis by inducing the expression of vascular endothelial growth factor (VEGF) [207, 208]. Novel therapeutic agents targeted to ERRα would be valuable to treat breast cancer.

Several ER-subtype selective SERMs have been reported, although it is difficult to design subtype selective ligands given the fact that only two amino acids are different in the ligand binding pocket between ERα and ERβ (despite that they have 61% amino acid identity in LBD). All the SERMs discussed previously were designed against ERα and have low subtype selection in terms of binding affinity. In contrast to the focus on ERα and breast cancer, most of ER subtype selective SERMs are developed for diseases other than breast cancer. In animal models, ERβ-selective agonists ERB041 and diaryl-propionitrile (DPN) have been shown to have anti-inflammatory properties and antidepressant-like effects, respectively [209, 210]. An ERβ agonist, 8-vinylestra-1,3,5 (10)-triene-3,17β-diol, stimulates ovarian follicular development in hypophysectomized rats and gonadotropin-releasing hormone a tagonist-treated mice [211], thus this drug could be used to enhance fertility [198]. A few ERβ agonists are being developed for clinical applications in Alzheimer's disease and rheumatoid arthritis [212]. For breast cancer prevention and treatment, it is conceivable that ERβ agonist might have potential for ERα-and-β-positive tumors, especially in combination of an ERα selective antagonist, since the preclinical studies indicate a protective role of ERβ. However, this strategy poses a difficult pharmacologic issue of tissue pharmacodynamics. Nevertheless, a couple of ERβ modulators have been shown with positive effects to treat advanced postmenopausal breast cancer, which will be discussed below.

4.4.1. Trilostane (Modrenal)

Trilostane (**17**) (Fig. (**6**)) is an inhibitor of 3β-hydroxysteroid dehydrogenase, a critical enzyme in the conversion of DHEA to estradiol in breast tumors [213]. Trilostane increases the maximum binding of estradiol to ERβ but not ERα in MCF-7 breast cancer cells [214], and it increased the expression of ERβ in MCF-7 cells and rat uterine [215]. Trilostane is approved in UK to treat advanced postmenopausal breast cancer after relapse to initial hormone therapy and is currently under investigation to be used in prostate cancer and premenopausal breast cancer [213].

4.4.2. TAS-108 (SR16234)

Another type of subtype selective SERM that might be relevant to breast cancer is a combined ERα antagonist but ERβ agonist. TAS-108 (**18**) (Fig. (**5**)) is a steroidal antiestrogen for ERα and a partial agonist on ERβ [216]. TAS-108 has pure antiestrogenic effects for ERα in the presence or absence of estrogen but exhibits partial agonist activity on ERβ using *in vitro* reporter assay. TAS-108 inhibits the growth of tamoxifen-resistant breast cancer cells, DMBA-induced mammary tumor in rats and estrogen-stimulated growth of MCF7 xenografts with little uterotrophic effect [216, 217]. Phase I trial indicate that TAS-108 has anti-tumor activity, is well tolerated, and does not have effects on an endometrial thickness based on an evaluation using trans-vaginal ultrasound [218, 219]. Similar results were obtained in Phase II trials that recruited postmenopausal women with advanced breast cancer, according to presentations at San Antonio Breast Cancer Symposium (SABCS) in December, 2008. A phase III trial is planned [217]. TAS-108 did not increase bone loss like fulvestrant, which could be due to its agonistic property on ERβ. Another advantage over fulvestrant is that TAS-108 is orally administered [220]. TAS-108 is therefore a promising breast cancer drug, even for patients who have relapsed after tamoxifen.

5. CONCLUDING REMARKS

Endocrine therapy targeting to ERα has been very successful in the treatment and prevention of breast cancer [221, 222]. It is very effective and less toxic compared to combinational cytotoxic chemotherapy that was the only option 30 years ago. In the ensuing period, multiple strategies have been developed to antagonize estrogen action. Most experience has accumulated with the competitive inhibitor of estrogen action tamoxifen, but targeting aromatase to deplete estrogen with AIs in postmenopausal patients or to induce ER degradation with SERDs have been valuable innovations in therapies. The goal for treatment is to create a "no-estrogen environment". However, SERMs that maintain the beneficial effects of estrogen but antagonize the harmful effects of estrogen have great potential in the prevention of multiple diseases in common. It is clear that many new SERMs are being developed that could provide better choices for patients in the future.

To overcome the unwanted side effects and problems with drug resistance, combination therapy might be another important direction in addition to the development of new SERMs. For example, combination of SERM acolbifene and DHEA could be protective against breast cancer and osteoporosis with beneficial effects to stimulate vaginal maturation and decrease skin dryness [182]. As traditional HRT is less acceptable to regulatory authorities because of the increased risk of breast cancer, a combination of HRT and a SERM may be a reasonable idea to relieve unpleasant menopausal effects while decrease breast cancer risks. With regards to avoiding drug resistance, combining a SERM and an inhibitor targeting significant survival signal transduction pathway is under active evaluation. By way of example, a combination of tamoxifen and inhibitors of the HER2 signal transduction pathway may prevent acquired tamoxifen resistance [223]. Similarly, SERM pipendoxifene and mTOR inhibitor temsirolimus synergistically inhibits the proliferation of MCF7 breast cancer cells and xenograft at suboptimal concentrations [168]. Additionally, combinations of a SERM (arzoxifene or acolbifene) and a rexinoid LG100268 are effective to prevent and treat ER-negative mammary tumors in animal models [155]. The potential combination seems endless but the marriage of molecular biology and medicine holds great promise for advances in targeted therapeutics based on the SERM model.

In summary, it is clear that the original idea of targeting specific hormone receptor with selective medicine has proven its worth by advancing medicine with the SERMs tamoxifen and raloxifene. Now there are a whole range of new SERMs poised for clinical applications. But this is not the end of the story. Novel selective modulators of all members of the nuclear receptor superfamily are under investigation addressing the treatment or prevention of diseases never before considered possible [57, 222].

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Table. Current status of new SERMs

Drug Name	Category (Structure)	Effects	Preclinical Results	Clinical Status
Toremifene	Tamoxifen-like	Breast cancer treatment	Fewer genotoxic effects than tamoxifen [113], bone effects similar to tamoxifen [119]	FDA approved for metastatic breast cancer
		Heart protection		Phase II trial (65 women) better than tamoxifen regulating lipid metabolism [121, 122]
		Mastalgia treatment		Phase II trials (62 and 195 women) effective [126, 127]
		Prostate cancer prevention		Phase II trial (514 men) decreases prostate cancer incidence [128]
		Relieve side effects of androgen deprivation therapy		Phase III trial (1,389 men) improves lipid profiles [130] Phase III trial (1,392 men) increases bone mineral density [129]
Ospemifene	Tamoxifen-like	Vaginal atrophy treatment	Estrogenic effects on vaginal epithelium that is not observed with tamoxifen or raloxifene [134-136]	Phase III trial (826 women) relieves vaginal dryness
		Osteoporosis treatment		Phase II trial (118 women): Comparable to or slightly better than raloxifene [139] Phase III trial planned (detail not available)
		Breast cancer prevention	Inhibits tumor growth in animal models as effective as tamoxifen [140, 141]	Not available
GW5638 (DPC974) & GW7604	Tamoxifen-like	Breast cancer treatment (2nd line therapy)	Works as a SERM and as a SERD [148], effective in tamoxifen-resistant tumors [144, 145]; functions as an ER agonist in bone and cardiovascular system but an antagonist in breast and endometrium [142]	Phase I trial (9 patients who failed first-line hormone therapy) low toxicity [ASCO meeting 2002, abstract 452]

(Continued)

Drug Name	**Category**	**Effects**	**Preclinical Results**	**Clinical Status**
Arzoxifene (LY353381)	Raloxifene-like	Breast cancer treatment	Antiestrogenic in breast and endometrium, estrogenic in bone and lipids [150]	Phase III trial (200 patients) inferior to tamoxifen [154]
		Breast cancer prevention	Effective to prevent ER-positive and ER-negative mammary tumors especially in combination with LG100268 [140, 155]	Phase I trials (50 and 76 women) low toxicity and favorable biomarker profile [156]
Lasofoxifene (CP-336156, Fablyn)	Raloxifene-like	Osteoporosis treatment and prevention	Higher potency than tamoxifen and raloxifene [158]; higher oral bioavailability than raloxifene [160]	Phase III trial (1,907 women) significantly increases bone mineral density compared to placebo, no endometrial effects, no association with thromboembolic disorder [159] Phase III trial to compare with raloxifene (CORAL trial, details not available)
		Vaginal atrophy treatment		Phase III trail (445 patients) improves vaginal atrophy compared to placebo
		Breast cancer treatment and prevention Heart disease prevention	Effects similar to tamoxifen to prevent and treat NMU-induced mammary tumor in rats [163]	Phase III trial (PEARL trial with 8,556 women), reduces ER-positive breast cancer incidence compared to placebo; slightly decreases major coronary disease risk; reduces vertebral and non-vertebral fractures; increases risks of venous thromboembolic events but not stroke; no endometrial effects [SABCS 2008, abstract 11]
Pipendoxifene (ERA-923)	Raloxifene-like	Breast cancer treatment	Inhibits tamoxifen-sensitive and -resistant tumors in mice and rats no uterotrophic activities compared to raloxifene [167]	Phase II trial to treat tamoxifen-refractory breast cancer in postmenopausal women (details not available)

(Continued)

Drug Name	**Category**	**Effects**	**Preclinical Results**	**Clinical Status**
Bazedoxifene (TSE-424 WAY-140424)	Raloxifene-like	Osteoporosis treatment and prevention	Increases bone density with little uterine or vasomotor effects	Phase III trial (7,492 women) reduces vertebral and non-vertebral fracture incidences, while raloxifene is not effective against non-vertebral fracture [171] Phase III trial (497 women) reduces endometrial thickness, unique property among known SERMs [170]
		Breast cancer prevention	Inhibits estrogen-stimulated breast cancer cells growth [169]	Not available
Acolbifene (EM-652, SCH57068) & EM-800 (SCH57050)	Raloxifene-like	Breast cancer treatment (2nd line therapy)	Highest affinity for ER, inhibit growth of multiple breast cancer cells *in vitro* and *in vivo* [180]	Phase III trial, less effective than anastrozole to treat tamoxifen-resistance breast cancer, study halted [182]
		Breast cancer treatment (1st line therapy)		Phase III trial planned [182]
		Breast cancer prevention		Phase II trial (started in February, 2009) for premenopausal women
CHF4227	Raloxifene-like	Breast cancer and osteoporosis prevention	Prevents DMBA-induced mammary tumors and preserves bone mass in rats [184, 185];	Phase I trials (24 and 56 women) beneficial on bone markers and lipid metabolism; no effects on endometrium; not causing hot flashes
SP500263	Raloxifene-like	Breast cancer and osteoporosis treatment	Inhibits breast cancer cell growth *in vitro* and *in vivo* without stimulating uterine weight gain [188, 189], protects bone *in vitro* [190]	Not available
HMR3339	Steroidal	Osteoporosis and cardiovascular disease prevention	Better than raloxifene to protect cancellous bones [191]	Phase II trials (96 and 118 and 94 women) better than raloxifene at improving some beneficial cardiovascular markers [192-194]
PSK3471	Steroidal	Osteoporosis and breast cancer prevention and treatment	Prevents bone loss *in vivo*, inhibits growth of breast cancer cells *in vivo* [197]	Not available

(Continued)

Drug Name	**Category**	**Effects**	**Preclinical Results**	**Clinical Status**
Trilostane (Modrenal)	ER subtype-selective	Breast cancer treatment	Increases estradiol binding to ERβ, increases ERβ expression, partially inhibits estrogen production [214, 215]	Approved in UK to treat advanced postmenopausal breast cancer after relapse to initial hormone therapy Phase III trial (714 women with advanced breast cancer) effective for both ER-positive and ER-negative breast cancer, effective for endocrine therapy-resistant cancer Phase II trial for use in premenopausal breast cancer (details not available)
		Prostate cancer treatment		Phase II trial with hormone-refractory prostate cancer (details not available)
TAS-108 (SR16234)	Steroidal, ER subtype-selective	Breast cancer treatment and prevention	Inhibits growth of tamoxifen- and AI-sensitive and resistant cancer cells *in vitro* and *in vivo*; inhibits DMBA-induced tumor growth in rats [216]	Phase I trials (16 and 15 women) effective and well tolerated [218, 219] Phase II trails (145 and 97 postmenopausal women with advanced breast cancer) beneficial effects, well tolerated [SABCS, 2008, abstract 2131and 2132]

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FIGURE LEGENDS

Fig. (**1**). Schematic comparison of human ER-α and ER-β structure. The structural domains are shown, and the percentage of amino acid identity shared by the two ERs is indicated for each domain. The horizontal bars highlight areas of different functions.

Fig. (**2**). Schematic representation of different liganded-ER complexes interacting with co-regulators and consequent transcriptional activities. ERs that bind to estrogenic ligands interact with co-activators (CoA) and activate transcription. Anti-estrogen liganded-ER complexes interact with co-repressors (CoR) and inactivate transcription of responsive genes. Selective estrogen receptor modulators (SERMs) bind to ERs and interact with either co-activator or co-repressor complexes eliciting partial transcriptional activity depending upon the cellular context.

Fig. (**3**). SERMs with a structure mimicking tamoxifen containing a triphenylethylene core.

Fig. (**4**). SERMs with a structure mimicking raloxifene.

Fig. (**5**). Steroidal SERMs

Fig. (**6**). Structure of ERβ-selective agonists.

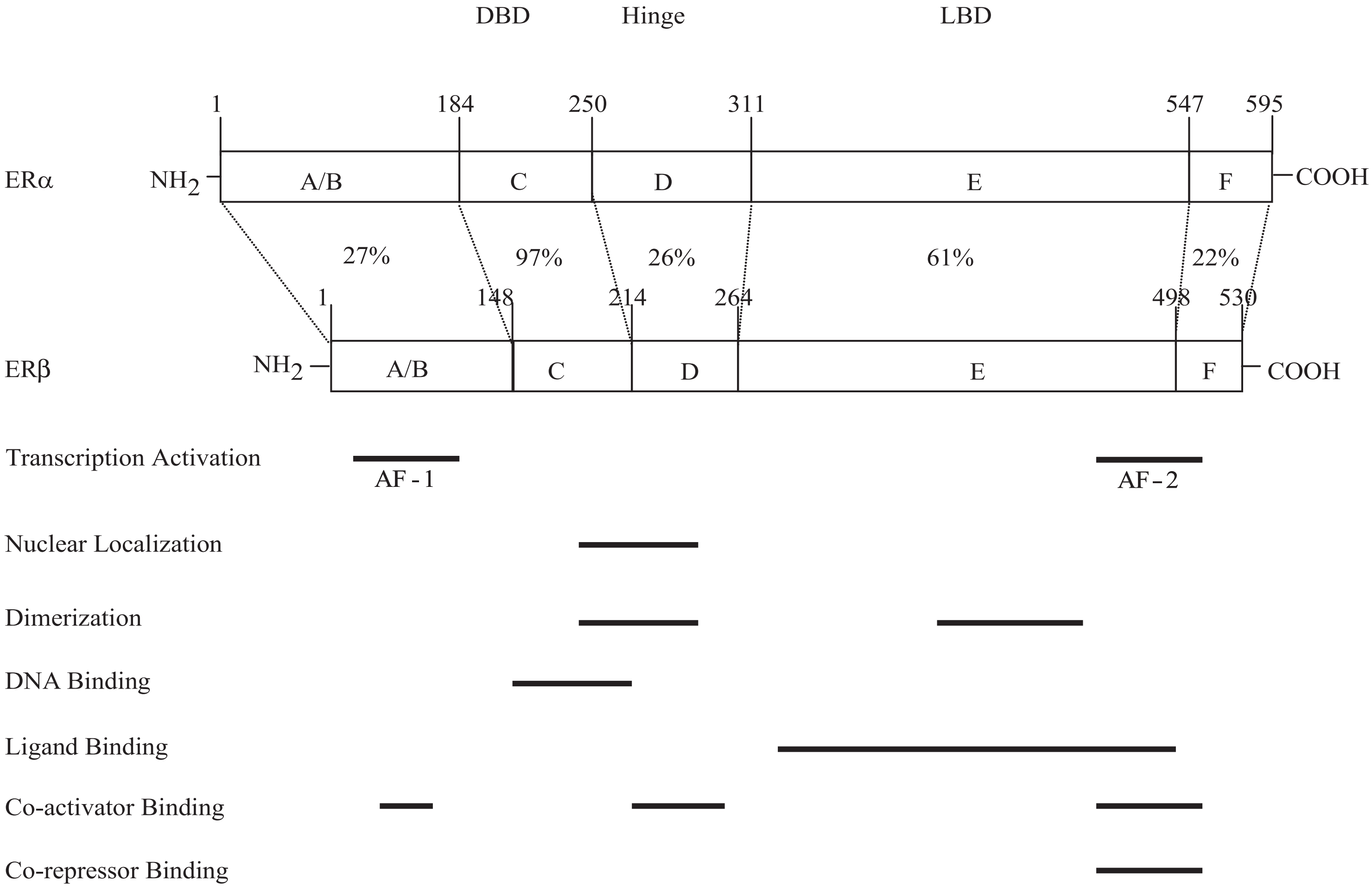

DBD
Hinge
LBD
1
184
250
311
547
595
ERα
NH2
A/B
C
D
E
F
COOH
27%
97%
26%
61%
22%
1
148
214
264
498
530
ERβ
NH2
A/B
C
D
E
F
COOH
Transcription Activation
AF-1
AF-2
Nuclear Localization
Dimerization
DNA Binding
Ligand Binding
Co-activator Binding
Co-repressor Binding

Fig. (**1**).

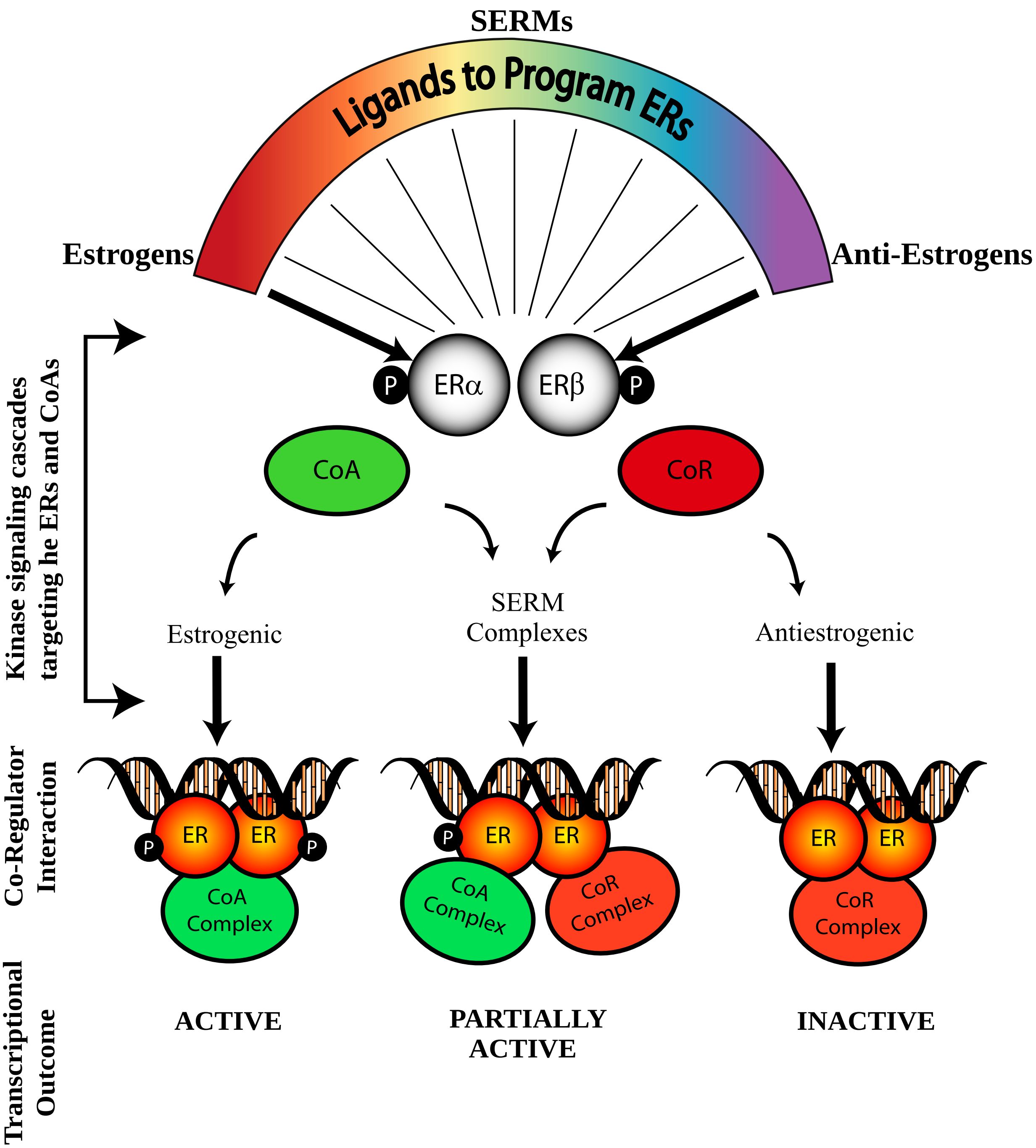
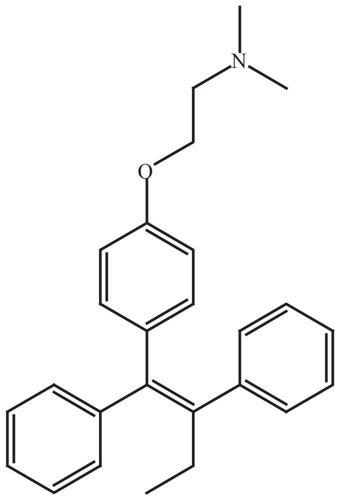
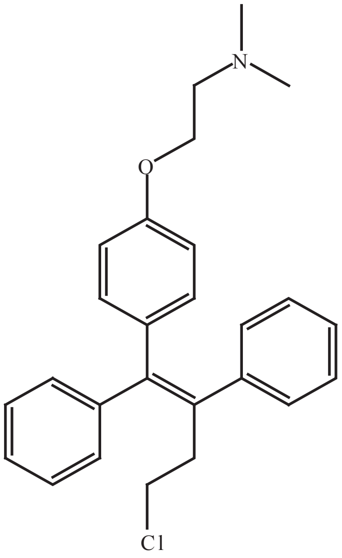

SERMs
Ligands to Program ERs
Estrogens
Anti-Estrogens
Kinase signaling cascades targeting he ERs and CoAs
P
ERα
ERβ
P
CoA
CoR
Estrogenic
SERM Complexes
Antiestrogenic
Co-Regulator Interaction
ER
ER
P
P
CoA Complex
P
ER
ER
CoA Complex
CoR Complex
ER
ER
CoR Complex
Transcriptional Outcome
ACTIVE
PARTIALLY ACTIVE
INACTIVE

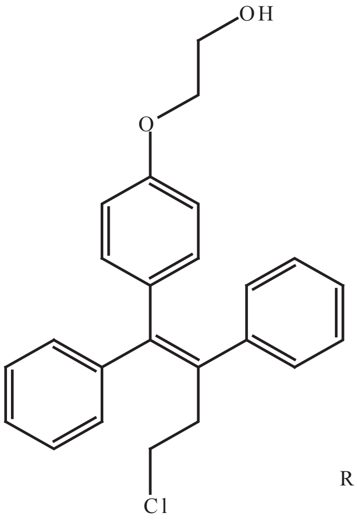
Fig. (**2**).



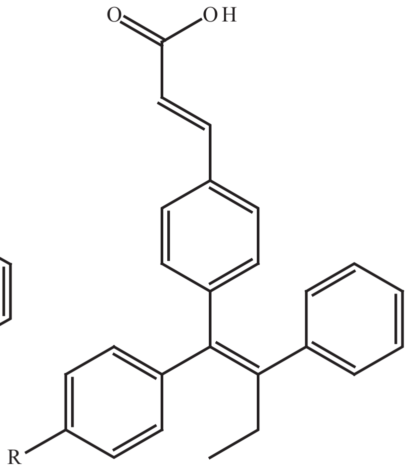
(**1**) Tamoxifen



(**2**) Toremifene



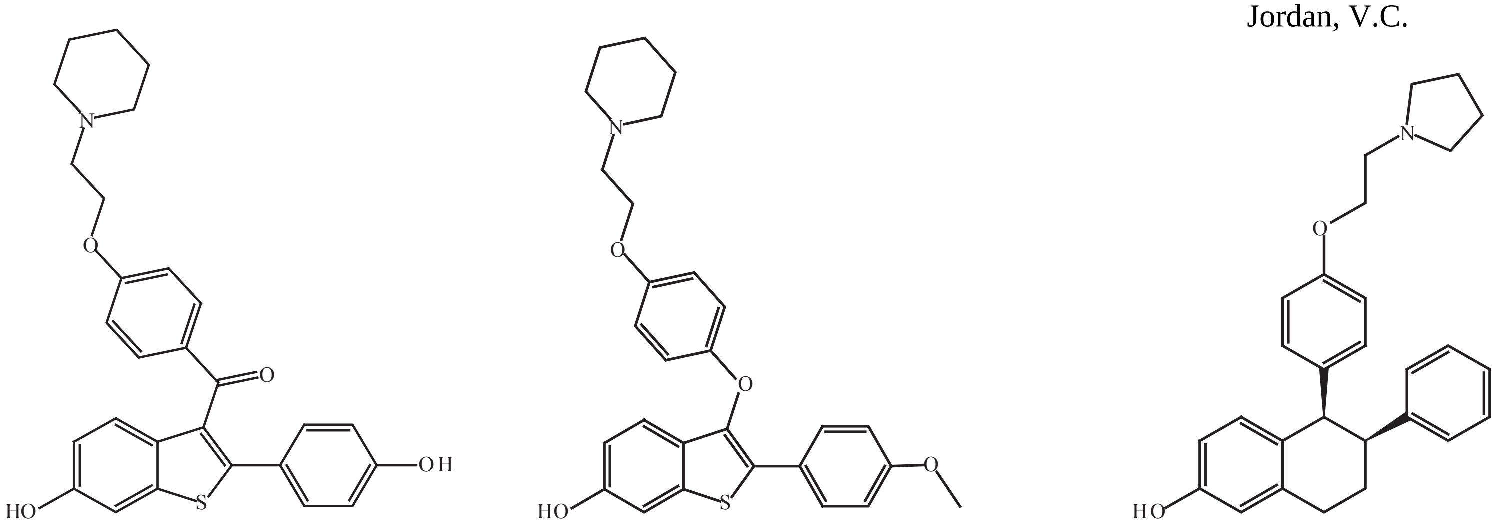
(**3**) Ospemifen



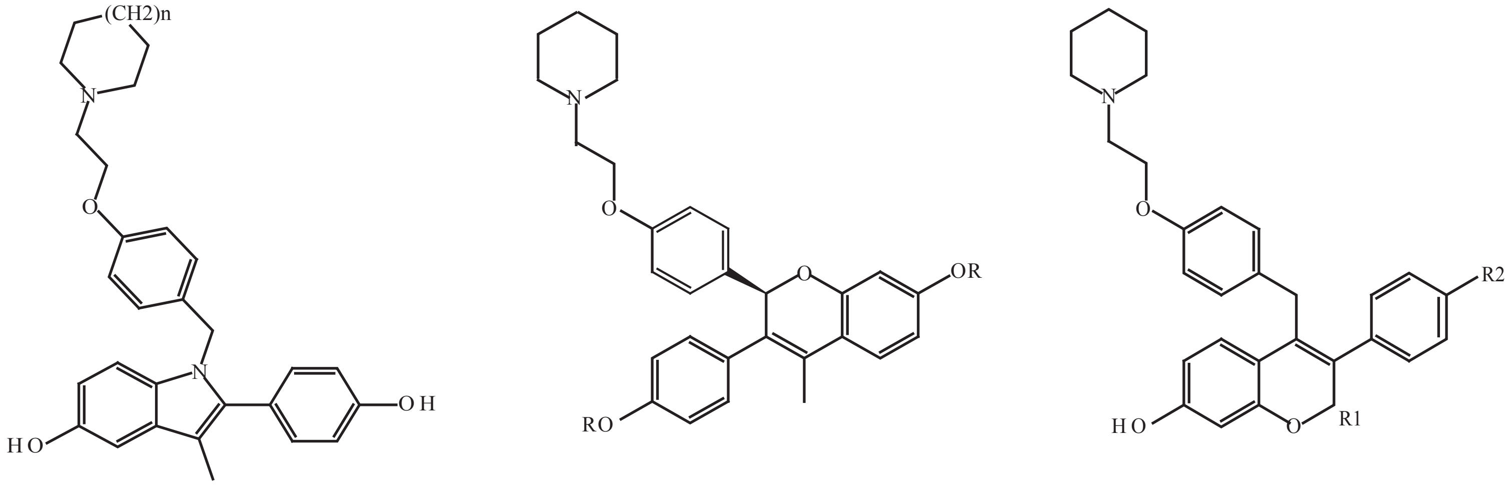
(**4**) GW5638 R=H

(**5**) GW7604 R=OH

Fig. (**3**).


N
O
O
OH
HO
S
N
O
O
O
HO
S
N
O
HO

(**6**) Raloxifene (**7**) Arzoxifene (**8**) Lasofoxifene


(CH2)n
N
O
N
OH
HO
N
O
O
OR
RO
N
O
R2
HO
O
R1

(**9**) Pipendoxifene n=1

(**10**) Bazedoxifene n=2

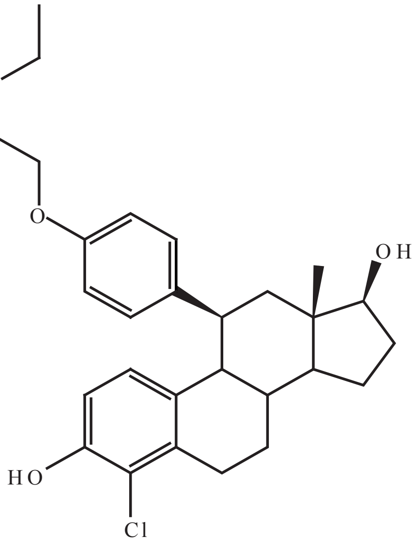
(**11**) Acolbifene (EM-652) R=H

(**12**) EM-800 R=COC(CH3)3

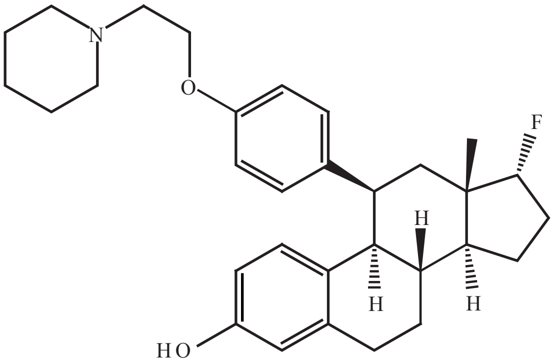
(**13**) CHF-4227 R1=H2; R2=O-CH3

(**14**) SP500263 R1= ═O; R2=H

Fig. (**4**).

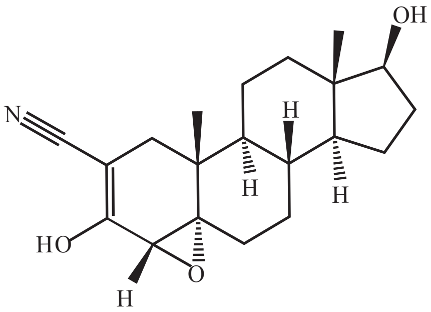


(**15**) HMR3339

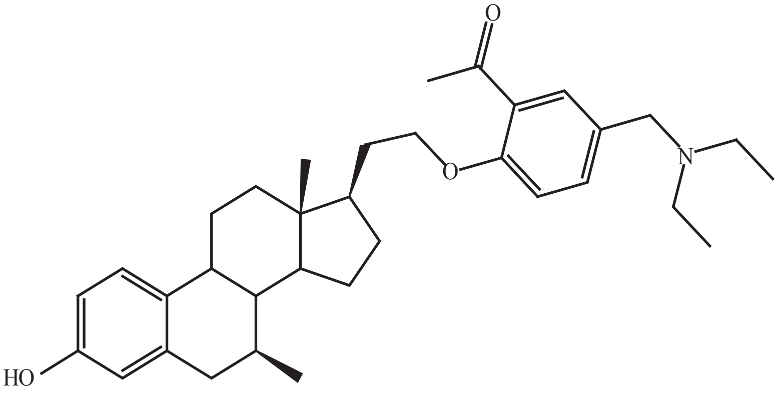


(**16**) PSK3471

Fig. (**5**).



(**17**) Trilostane



(**18**) TAS-108 (SR16234)

Fig. (**6**).

Jordan, V.C.

Review

Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit?

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Abstract

The link between estrogen and the development and proliferation of breast cancer is well documented. Estrogen stimulates growth and inhibits apoptosis through estrogen receptor-mediated mechanisms in many cell types. Interestingly, there is strong evidence that estrogen induces apoptosis in breast cancer and other cell types. Forty years ago, before the development of tamoxifen, high-dose estrogen was used to induce tumor regression of hormone-dependent breast cancer in post-menopausal women. While the mechanisms by which estrogen induces apoptosis were not completely known, recent evidence from our laboratory and others demonstrates the involvement of the extrinsic (Fas/FasL) and the intrinsic (mitochondria) pathways in this process. We discuss the different apoptotic signaling pathways involved in E2 (17β-estradiol)-induced apoptosis, including the intrinsic and extrinsic apoptosis pathways, the NF-κB (nuclear factor-kappa-B)-mediated survival pathway as well as the PI3K (phosphoinositide 3-kinase)/Akt signaling pathway. Breast cancer cells can also be sensitized to estrogen-induced apoptosis through suppression of glutathione by BSO (L-buthionine sulfoximine). This finding has implications for the control of breast cancer with low-dose estrogen and other targeted therapeutic drugs.

Introduction

Breast cancer is one of the most frequently diagnosed cancers among women, with an estimated 184,450 new cases of invasive disease and 40,930 deaths in 2008. There is strong evidence that estrogen plays a role in its development and progression [1]. Breast cancer was first recognized to be estrogen-dependent when the British surgeon George Beatson [2] published his findings of the beneficial effects of oophorectomy in a pre-menopausal patient with advanced breast cancer. Beatson had based his approach on the role of the ovaries in mammalian lactation and presumed that there would be a similar mechanism for breast cancer growth. Since that time, there has been an expanding clinical database that implicates estrogen in the development and progression of breast cancer. Evidence to support this conclusion comes from clinical studies of hormone replacement therapy, which were initially designed to determine the benefits of replacement approaches on post-menopausal women's health [3,4], and the successful clinical strategy of treating breast cancer by blocking estrogen action using the anti-estrogen tamoxifen [5] or preventing estrogen synthesis using aromatase inhibitors (AIs) [6].

Estrogens are a class of sex steroid hormones that are synthesized from cholesterol and are secreted primarily by the ovaries, with secondary contributions from placenta, adipose tissue, testes, and adrenal glands. After menopause, ovarian estrogen biosynthesis is minimal, with circulating estrogens being derived principally from peripheral aromatization of adrenal androgens. Estrogens are essential to the function of the female reproductive system and are required for the proliferation and differentiation of healthy breast epithelium. Estrogens occur naturally in several structurally related forms; however, the predominant intracellular estrogen is 17β-estradiol (E2). In mammary glands, E2 promotes cell proliferation in both normal and transformed epithelial cells by modifying the expression of hormone-responsive genes involved in the cell cycle and/or programmed cell

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AI = aromatase inhibitor; AP-1 = activator protein 1; Bad = Bcl-2/Bcl-X_L-associated death domain protein; Bak = Bcl-2 homologous antagonist-killer protein; Bax = Bcl-2-associated X protein; Bcl-2 = B-cell lymphoma-2; Bcl-X_L = Bcl-2-related gene, long form; BH = Bcl-2 (B-cell lymphoma-2) homology; Bid = Bcl-2-interacting domain; Bim = Bcl-2-interacting mediator of cell death; BSO = L-buthionine sulfoximine; CDK = cyclin-dependent kinase; CR = complete response; DES = diethylstilbestrol; E2 = 17β-estradiol; ER = estrogen receptor; FasL = Fas ligand; GSH = glutathione; IL = interleukin; JNK = c-jun N-terminal kinase; LTED = long-term estrogen-deprived; MAPK = mitogen-activated protein kinase; NF-κB = nuclear factor-kappa-B; OPG = osteoprotegerin; p53 = 53 kDa protein; PARP = poly(ADP-ribose)polymerase; PI3K = phosphoinositide 3-kinase; PKB/Akt = protein kinase B; PKC-α = protein kinase C-alpha; Puma = p53-upregulated modulator of apoptosis; RANK-L = ligand of the receptor activator of nuclear factor-kappa-B; SERM = selective estrogen receptor modulator; siRNA = short interfering RNA; Sp-1 = specificity protein 1; TNF = tumor necrosis factor.

death. In estrogen receptor (ER)-positive MCF-7 human breast cancer cells, one of the principal mechanism by which E2 stimulates growth is through the induction of G_1- to S-phase transition. This induction is associated with the rapid and direct upregulation of c-myc, which controls cyclin D1 expression along with activation of cyclin-dependent kinase (CDK) and phosphorylation of retinoblastoma protein [7]. E2 also rapidly activates cyclin E-CDK2 complexes, accelerating the G_1-to-S transition [8]. Additionally, E2 has ‘non-genomic or membrane-initiated’ effects (that is, independent of ER-mediated transcription) that occur within minutes after E2 administration [9-11]. Specifically, ER-α interacts with a number of proteins, including c-Src, the p85 subunit of phosphoinositide 3-kinase (PI3K), caveolin 1, and modulator of non-genomic activity of ER (MNAR) [10,12], epidermal growth factor receptor (EGFR), insulin-like growth factor receptor 1 (IGFR1), and HER2 [13], and it rapidly increases PIP2-phospholipase C activity and activates the mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways [9,12,13]. More importantly, E2 is a potent inhibitor of apoptosis and it regulates the expression of several apoptotic proteins, including Bcl-2 in MCF-7, T47-D, and ZR-75-1 breast cancer cells [14].

Remarkably, there is another dimension to estrogen action which contrasts with its ability to stimulate growth and inhibit apoptosis. Physiologic E2 is also capable of inducing apoptosis in breast cancer cells that have been long-term estrogen-deprived (LTED) or have been treated exhaustively with anti-estrogens [15-23], prostate cancer cells [24], neuronal cells [25], bone-derived cells [26], thymocytes [27], and ER-transfected cells [28,29]. These data are particularly interesting because high-dose estrogen therapy was used as a treatment for post-menopausal patients with metastatic breast cancer from the 1940s until the introduction of the safer anti-estrogen tamoxifen in the 1970s [30]. At that time, however, the mechanism of estrogen-induced tumor regression was not known. In this review, we will discuss the current understanding of estrogen-induced apoptosis in breast cancer and will summarize the possible mechanisms involved in this estrogen-mediated process.

Estrogen-induced apoptosis: laboratory observations

Recent *in vitro* studies from our laboratory [18,31] and other investigators [19,20,32] have shown that long-term estrogen deprivation of hormone-dependent MCF-7 breast cancer cells causes them to undergo adaptive changes in which estradiol switches from being a proliferative agent to paradoxically inhibiting growth and inducing apoptosis. Interestingly, LTED cells also exhibit enhanced sensitivity to estradiol in that an estradiol concentration that is three logs lower can stimulate proliferation of these cells compared with wild-type MCF-7 cells [19]. The development of hypersensitivity to estradiol as a result of LTED is associated with the upregulation of ER-α and the MAPK, PI3K, and mTOR (mammalian target of rapamycin) growth factor pathways [33]. In contrast, the apoptotic mechanisms of estradiol in LTED cells are thought to involve the death receptors as well as the mitochondrial pathways. Specific molecular events include the activation of the Fas death receptor/Fas ligand (FasL) complex [20], the release of cytochrome *c* from the mitochondria and alterations in Bcl-2 [18,32], and the downregulation of the anti-apoptotic factor nuclear factor-κ [31,34]. It is important to note that estradiol also induces apoptosis in *in vivo* models of anti-hormone drug resistance [23,35,36]; however, the mechanisms by which this occurs are not completely known.

Estrogen therapy and breast cancer: clinical observations

Clinical data support the use of high-dose estrogen to treat hormonally sensitive breast tumors [37-41]. In 1944, Sir Alexander Haddow and colleagues [37] published the results of their clinical trial with the synthetic estrogens triphenylchlorethylene, triphenylmethylethylene, and stilbestrol administered at high doses. They found that 10 out of 22 post-menopausal patients with advanced mammary carcinomas, who were treated with triphenylchlorethylene, had significant regression of tumor growth. Five patients out of 14 who were treated with high-dose stilbestrol produced similar responses. Interestingly, the duration of the post-menopausal period was found to be a critical factor affecting the success of this therapy. For example, when the synthetic estrogen diethylstilbestrol (DES) was administered at 15 mg per day, women who had experienced the onset of menopause less than 1 year prior to therapy did not respond to DES; women who had experienced the onset of menopause within 5 years of menopause experienced a 7.9% objective response rate; and women who reached menopause more than 10 years earlier experienced a 22% response rate [41]. Despite the benefits, however, there were significant systemic side effects associated with high-dose estrogen therapy [37].

Cole and colleagues [39] reported the first clinical trial of the anti-estrogen tamoxifen in women with late or recurrent breast cancer and compared their findings with historical data from women receiving DES. They concluded that the levels of response were similar for DES and tamoxifen; however, tamoxifen had a lower incidence of side effects. Ingle and colleagues [30] compared tamoxifen with DES directly and noted that response rates were similar but tamoxifen had fewer side effects. Based on these data, the use of high-dose estrogen for treatment of advanced breast cancer fell out of favor, and tamoxifen became the standard first-line endocrine therapy. The Ingle study [30] that compared DES-treated and tamoxifen-treated patients was followed up but surprisingly showed a survival advantage for DES-treated patients [41]. Another small trial was conducted by Lonning and colleagues [40] in post-menopausal patients with advanced breast cancer exposed to multiple endocrine therapies and revealed a 31% objective response rate with DES therapy. More recently, Ellis and colleagues [42] reported that a daily dose of 6 mg of E2 could stop the growth of tumors or even cause them to shrink in about 25% of women with metastatic breast cancer that had developed resistance to standard anti-hormonal therapy. These clinical observations that estrogen can induce tumor regression after several years of anti-hormonal therapy provide a clue that the adaptation of cancer cells to low levels of estrogen might sensitize cells to the apoptotic effect of estrogen. While the mechanisms by which estrogen exerts its pro-apoptotic/anti-tumor effect are not known, a growing body of evidence suggests the involvement of the extrinsic (death receptor) and intrinsic (mitochondrial) pathways in this process.

Two main pathways involved in apoptosis regulation

Apoptosis is a form of programmed cell death that plays a critical role in the maintenance of tissue homeostasis [43]. It is a highly regulated physiologic mechanism that removes excess or damaged cells [43]. The dysregulation of apoptosis is a hallmark of cancer, with both the loss of pro-apoptotic signals and the gain of anti-apoptotic mechanisms contributing to tumorigenesis [44]. The induction of apoptosis in many cell types is achieved through the activation of the extrinsic and the intrinsic pathways [45]. The extrinsic pathway (Figure 1) is initiated by the interaction between specific ligands and surface receptors, such as CD95/Fas/Apo1, tumor necrosis factor (TNF) receptor 1 (TNFR1), TNF receptor 2 (TNFR2), and death receptors 3-6 (DR3-6) [46], which are able to deliver a death signal from the extracellular microenvironment to the cytoplasm. Binding of the ligand to the receptor induces receptor multimerization, binding of Fas-associated death domain (FADD) adapter protein, formation of the death-induced signaling complex (DISC) which recruits the initiator caspases 8 and 10, and subsequently activation of the effector caspases 3 and 7 [46]. In the intrinsic pathway (Figure 1), the integrity of the mitochondrial membrane is controlled primarily by a balance between the antagonistic actions of the proapoptotic and antiapoptotic members of the Bcl-2 family [47] (please see Table 1 for a detail description of common abbreviations used in apoptosis). Bcl-2 family proteins comprise three principal subfamilies: (a) anti-apoptotic members, including Bcl-2/Bcl-x_L, which possess the Bcl-2 homology (BH) domains BH1, BH2, BH3, and BH4; (b) pro-apoptotic members, such as Bax, Bak, and Bok, which have the BH1, BH2, and BH3 domains; and (c) BH3-only proteins, such as Bid, Bim, Bad, Bik, and Puma, which generally possess only the BH3 domain [47]. The Bcl-2 family of proteins regulates apoptosis by altering mitochondrial membrane permeabilization and controlling the release of cytochrome *c*. Several lines of evidence demonstrate that the Bcl-2 family functions are controlled by growth factor signaling pathways, including the PI3K/Akt, the JAK (Janus kinase)/Stat (signal transducer and activator of transcription), and the Ras/MAPK pathways [48]. Phosphorylation and dephosphorylation of the members of the Bcl-2 family of proteins by the above pathways regulate the stabilization of mitochondrial homeostasis [48].

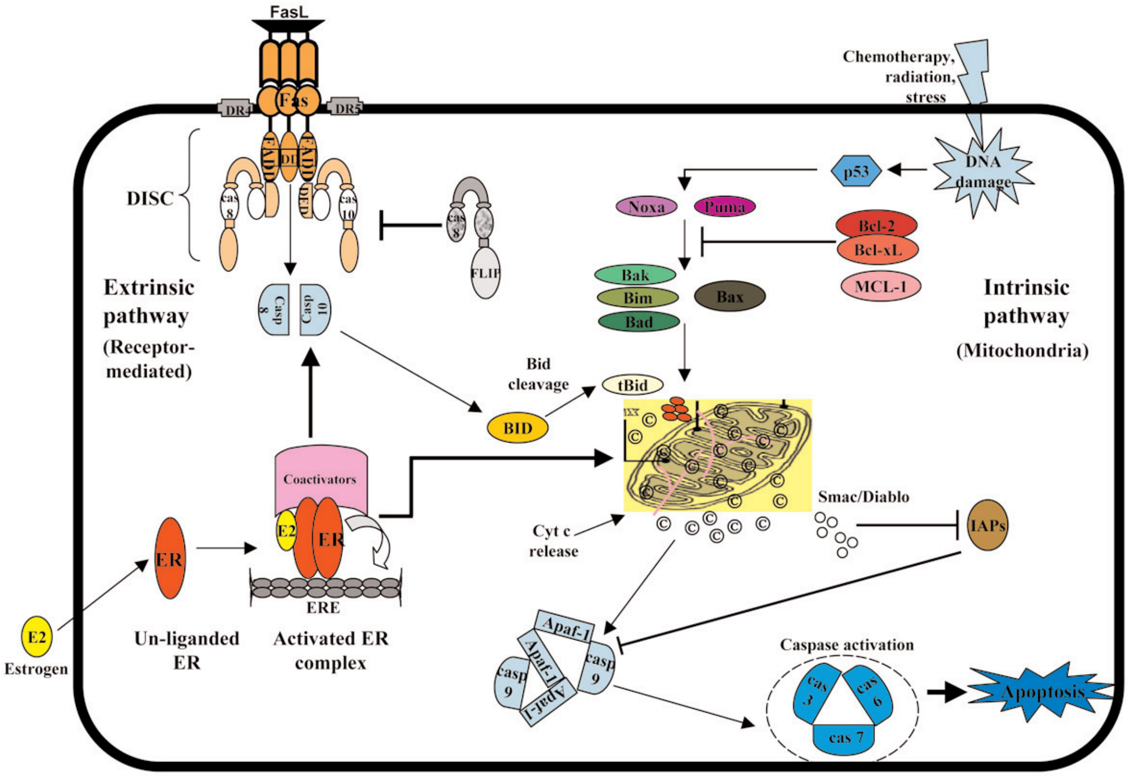
Signaling pathways implicated in estrogen-induced apoptosis

The extrinsic (receptor-mediated) pathway

Mechanistic studies have used either LTED MCF-7 breast cancer cells [18,20,31,34] or selective ER modulator (SERM) (tamoxifen or raloxifene)-stimulated tumor models [23,35,36,49-51] to demonstrate the involvement of the Fas/FasL death signaling pathway in the paradoxical apoptotic/anti-tumor effects of E2. Song and colleagues [20] were the first to demonstrate that E2 caused apoptosis in breast cancer cells that were adapted to grow in an E2-free environment for prolonged periods. They reported that their LTED cells, which were derived by growing wild-type MCF-7 breast cancer cells under long-term (6 to 24 months) estrogen-deprived conditions, expressed high levels of Fas compared with the parental MCF-7 cells and that treatment of these cells with E2 resulted in a marked increase in FasL. This finding was confirmed by Osipo and colleagues [35], who reported that physiologic levels of E2 induced regression of tamoxifen-resistant breast cancer tumors by inducing Fas expression and suppressing the anti-apoptotic/pro-survival factors nuclear factor-kappa-B (NF-κB) and HER2/neu. A similar finding was reported by Liu and colleagues [49] in raloxifene-resistant MCF-7 cells *in vitro* and *in vivo*. In addition, Tonetti and colleagues [50,51] previously reported that stable overexpression of protein kinase C-alpha (PKC-α) in hormone-responsive T47D:A18 breast cancer cells (T47D:A18/PKC-α) produced a hormone-independent/tamoxifen-resistant and E2-inhibitory phenotype *in vivo* [50,51]. Using the T47D:A18/PKC-α-overexpressing tumor model, they further demonstrated that E2-induced regression and apoptosis were due to increased expression of Fas/FasL proteins and downregulation of the pro-survival Akt pathway [36]. In all of these model systems, the ER-α was shown to be critical for E2-induced tumor regression and apoptosis. Blockade of the ER-α signaling pathway using the pure anti-estrogen fulvestrant completely inhibited the apoptotic effect of E2 [20,35,36,49].

It is worth noting that a putative estrogen-responsive element (ERE) has been identified in the promoter region of the FasL gene [52], suggesting direct estrogen effects on FasL expression. In addition, a number of transactivating factors have been identified as regulators of FasL gene expression, including activator protein 1 (AP-1) [53] and specificity protein 1 (Sp-1) [54]. Sp-1 is involved in the transcriptional regulation of many genes and has also been identified to be important in the regulation of FasL gene expression and apoptosis. Indeed, this transcription factor is able to activate FasL promoter via a distinct recognition element, and inducible FasL promoter activation is abrogated by the expression of the dominant-negative mutant form of Sp-1 [54]. Functional studies have demonstrated that Sp-1 is a crucial effector of E2 signal in enhancing FasL gene expression. For instance, it is well known that ERs can transactivate gene promoters without directly binding to DNA

Figure 1


FasL
Fas
DR4
DR5
FADD
DD
FADD
DED
DISC
cas 8
cas 10
cas 8
FLIP
Casp 8
Casp 10
Extrinsic pathway
(Receptor-mediated)
Chemotherapy, radiation, stress
DNA damage
p53
Noxa
Puma
Bcl-2
Bcl-xL
MCL-1
Bak
Bim
Bad
Bax
Intrinsic pathway
(Mitochondria)
Bid cleavage
tBid
BID
Coactivators
E2
ER
ERE
Cyt c release
Smac/Diablo
IAPs
Apaf-1
casp 9
Caspase activation
cas 3
cas 6
cas 7
Apoptosis
E2
Estrogen
ER
Un-liganded ER
Activated ER complex

The two main pathways involved in apoptosis regulation. **(a)** The extrinsic pathway begins outside the cell through the activation of receptors on the cell surface by specific molecules known as pro-apoptotic ligands, including CD95L/FasL (receptor CD95/Fas). Once activated, the death domains of these receptors bind to the adapter protein Fas-associated death domain (FADD), resulting in the assembly of death-induced signaling complex (DISC) and recruitment and assembly of initiator caspases 8 and 10. The two caspases are stimulated and processed, releasing active enzyme molecules into the cytosol, where they activate caspases 3, 6, and 7, thereby converging on the intrinsic pathway. **(b)** The intrinsic (mitochondrial) pathway is initiated in response to cellular signals resulting from DNA damage, a defective cell cycle, detachment from the extracellular matrix, hypoxia, loss of cell survival factors, or other types of severe cell stress. This triggers activation of specific members of the pro-apoptotic Bcl-2 protein family involved in the promotion of apoptosis, Puma and Noxa, which in turn activate the pro-apoptotic proteins Bax or Bak. These two proteins move to the mitochondrial membrane and disrupt the anti-apoptotic function of the Bcl-2 family proteins, allowing for permeabilization of the mitochondrial membrane. Apaf-1, apoptotic protease activating factor 1; Bad, Bcl-2/Bcl-X_L-associated death domain protein; Bak, Bcl-2 homologous antagonist-killer protein; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma-2; Bcl-X_L, Bcl-2-related gene, long form; Bid, Bcl-2-interacting domain; Bim, Bcl-2-interacting mediator of cell death; Casp, caspase; Cyt c, cytochrome *c*; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen-responsive element; FasL, Fas ligand; FLIP, FLICE-inhibitory protein; IAP, inhibitor of apoptosis; Noxa, phorbol-12-myristate-13-acetate-induced protein 1; Puma, p53-upregulated modulator of apoptosis.

but instead through interaction with other DNA-bound factors in promoter regions lacking TATA box. This has been investigated extensively in relation to protein complexes involving Sp-1 and ER-α at GC boxes, which are classic binding sites for members of the Sp-1 family of transcription factors. Sp-1 protein plays an important role in the regulation of mammalian and viral genes, and recent results have shown that E2 responsiveness of c-fos, cathepsin D, retinoic acid, and insulin-like grow factor-binding protein 4 gene expression in breast cancer cells is linked to specific GC-rich promoter sequences that bind ER/Sp-1 complex in which only Sp-1 protein binds DNA [55-59]. Thus, it is possible that, when E2 upregulates FasL production in these different model systems, an apoptotic signal is initiated by FasL binding on Fas receptor.

The intrinsic (mitochondrial) pathway

Over the last several years, there has been accumulating evidence that, apart from the extrinsic/receptor-mediated pathway, the mitochondrial (intrinsic) pathway plays a role in E2-induced apoptosis. Indeed, we [18] have previously reported that, in our LTED breast cancer cell line, MCF-7:5C, E2 treatment markedly increased the expression of several pro-apoptotic proteins, including, Bax, Bak, Bim, Noxa, Puma, and p53, and that blockade of Bax and Bim expression using short interfering RNAs (siRNAs) almost completely reversed

Table 1

Description of common abbreviations used in apoptosis and signal transduction

Abbreviation	Meaning	Synonyms
Bad	Bcl-2/Bcl-X$_L$-associated death domain protein	BH3-only member of the Bcl-2 family
Bak	Bcl-2 homologous antagonist-killer protein	Multi-BH domain pro-apoptosis protein
Bax	Bcl-2-associated X protein	Multi-BH domain pro-apoptosis protein
Bcl-2	B-cell lymphoma-2	Defining member of the family; originally characterized as an oncogene
Bcl-X$_L$	Bcl-2-related gene, long form	Bcl-X$_S$ is a shorter splice variant that is pro-apoptotic
Bim	Bcl-2-interacting mediator of cell death	BH3-only member of the Bcl-2 family
IκB	Inhibitor of NF-κB	Interacts with NF-κB
IKK	IκB kinase	Phosphorylates IκB to promote its degradation
MDM2	Murine double minute	Negative regulator of the p53 tumor suppressor
NF-κB	Nuclear factor-kappa type B	Originally linked with enhancement of immunoglobulin kappa light-chain gene
p53	53 kDa protein	Tumor-suppressor protein
PDK-1	3-phosphoinositide-dependent protein kinase 1	Master kinase that is crucial for the activation of Akt/PKB
PI3K	Phosphoinositide 3-kinase	Phosphatidylinositol 3-kinase; PI 3-kinase; PtdIns3K
PKB	Protein kinase B	Akt; RACK (related to A and C kinase); has PH domain
PMAIP-1/Noxa	Phorbol-12-myristate-13-acetate-induced protein 1	BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis
PUMA	p53-upregulated modulator of apoptosis	BH3-only member of the Bcl-2 family

BH, Bcl-2 (B-cell lymphoma-2) homology.

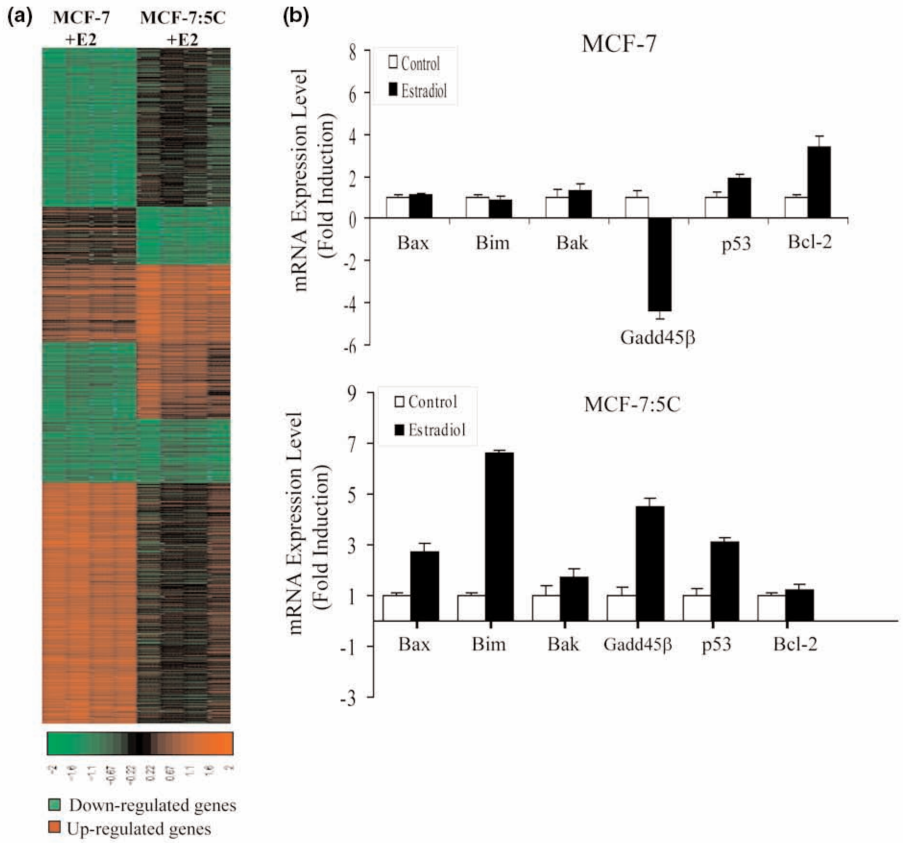
the apoptotic effect of E2 in these cells. E2 treatment also led to a loss of mitochondrial potential and a dramatic increase in the release of cytochrome *c* from the mitochondria, which resulted in activation of caspases 7 and 9 and cleavage of poly(ADP-ribose)polymerase (PARP). Furthermore, overexpression of anti-apoptotic Bcl-x$_L$ completely blocked E2-induced apoptosis in MCF-7:5C cells. Interestingly, microarray analysis of wild-type MCF-7 cells and LTED MCF-7:5C cells revealed significant differences in the gene expression profile between the two cell lines following E2 treatment (Figure 2a). In particular, E2 treatment caused a marked increase in several pro-apoptotic genes in MCF-7:5C cells compared with wild-type MCF-7 cells (Figure 2b).

In two other estrogen-deprived breast cancer lines, LTED and E8CASS, basal Bcl-2 level was greatly elevated and knock-down of Bcl-2 expression with siRNA markedly sensitized these cells to the apoptotic action of E2 [32]. A similar finding was reported for another LTED breast cancer cell line, MCF-7:2A, which expressed elevated basal levels of Bcl-2 and was initially resistant to E2-induced apoptosis [34]. We found that suppression of Bcl-2 expression in these cells enhanced the apoptotic effect of E2 by almost fivefold [34], thus suggesting an important role for this protein in the apoptotic action of E2. Currently, there is renewed interest in developing small-molecule inhibitors of Bcl-2 [60] as anti-cancer cell and anti-angiogenic agents. The Bcl-2 antisense oligonucleotide, Oblimersen (Genasense; Genta Incorporated, Berkeley Heights, NJ, USA), which works by blocking Bcl-2 protein production, is now in a phase III clinical trial [61].

Inhibition of the survival pathways Akt and nuclear factor-kappa-B

The existence of various checkpoints in apoptosis reveals a complex balance between cell survival and cell death in cells. Two of the main signaling pathways involved in cell survival are the Akt and NF-κB signaling pathways (Figure 3). The PI3K/Akt/protein kinase B (PKB) pathway plays a central role in a variety of cellular processes, including cell growth, proliferation, motility, and survival in both normal and tumor cells. It impinges upon a remarkable array of intracellular events that influence either directly or indirectly whether a cell will undergo apoptosis. Many of the transforming events in breast cancer are a result of enhanced signaling of the PI3K/Akt pathway. Akt, also called PKB, is the human homologue of the viral oncogene v-akt [62], which regulates multiple targets, including several apoptotic genes. In a series of publications [63,64], Akt was found to mediate phosphorylation and hence inactivation of pro-apoptotic factors like Bad, which controls the release of cytochrome *c*,

Figure 2



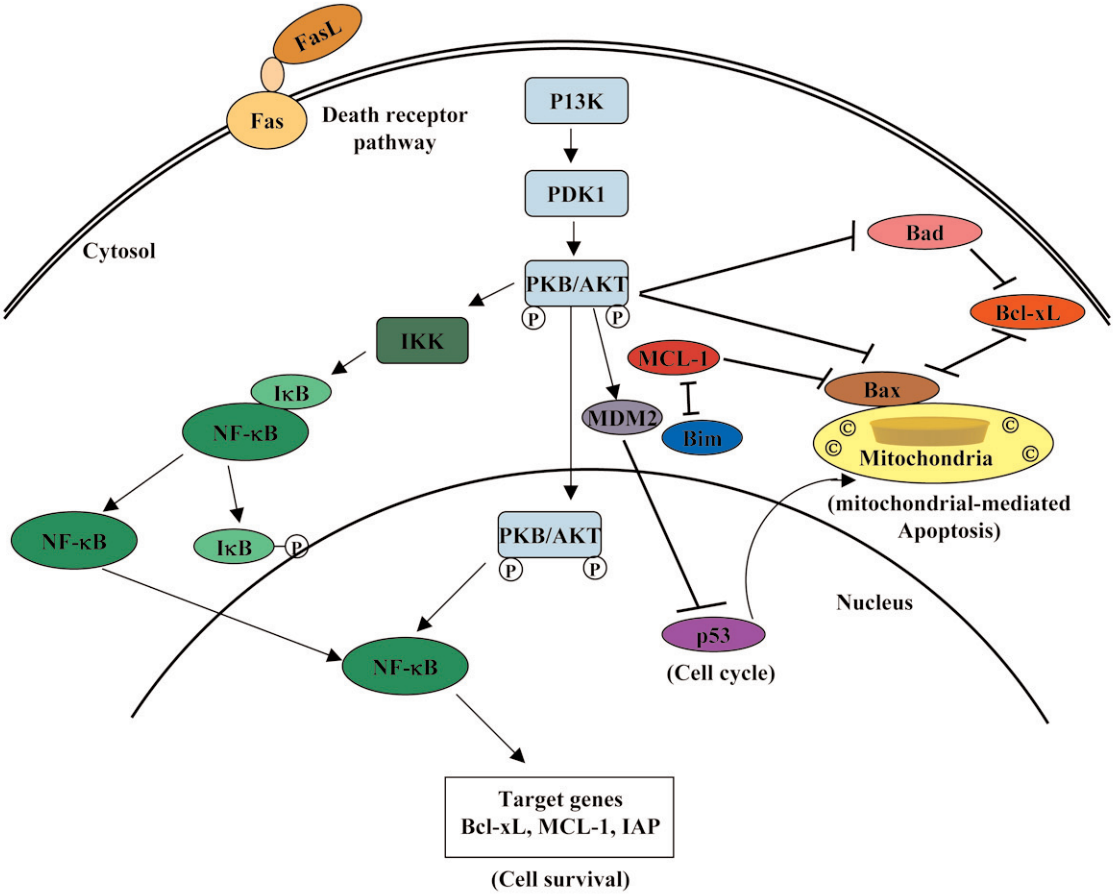
Gene expression profile of wild-type MCF-7 cells and long-term estrogen-deprived (LTED) MCF-7:5C breast cancer cells following 17β-estradiol (E2) treatment. Cells were treated with 1 nM E2 for 48 hours, and RNA was hybridized to the Affymetrix Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA, USA). **(a)** Hierarchical clustering dendogram of E2-regulated genes in MCF-7 and MCF-7:5C cells. Microarray expression data for each cell line were first filtered for minimal intra-replicate standard deviation (<0.25) and a standard deviation between all samples of at least 0.25. This generated a total of 2,743 genes. In addition, genes displaying a minimum of twofold upregulation or downregulation by E2 in either MCF-7 or MCF-7:5C cells were extracted, revealing a set of 539 differentially expressed, E2-regulated genes. **(b)** E2 regulation of pro-apoptotic and anti-apoptotic genes in MCF-7 cells (top panel) and MCF-7:5C cells (bottom panel). Bak, Bcl-2 homologous antagonist-killer protein; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma-2; Bim, Bcl-2-interacting mediator of cell death; GADD45β, growth arrest and DNA damage; p53, 53 kDa protein.

procaspase 9, and Forkhead transcription factors. Akt also activates anti-apoptotic genes, including cyclic-AMP response element-binding protein (CREB) and IκB (inhibitor of NF-κB) kinase (IKK), the primary regulator of NF-κB activity.

Several groups have demonstrated that E2 can also inhibit the P13K/Akt signaling pathway and consequently induce apoptosis of cancer cells. In tamoxifen-resistant PKC-α-overexpressing cells, E2-induced tumor regression is associated with the downregulation of phosphorylated Akt [36]. In addition, in LTED MCF-7:5C and MCF-7:2A breast cancer cells, the basal level of phosphorylated Akt is markedly upregulated and E2 treatment significantly reduces its expression (Figure 4). There is also evidence that, in MCF-7.beclin-overexpressing cells, E2 treatment significantly reduces Akt phosphorylation, which is associated with a decrease in cell proliferation [65]. Akt, therefore, is considered a rational target for cancer therapies and inhibitors of the PI3K/Akt pathway have been identified [66].

NF-κB is one of the most studied transcription factors in mammalian cells. Its family is composed of five members: RELA (p65), RELB, REL (cRel), NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100) [67]. These proteins form homodimeric and heterodimeric complexes, and the activity of these proteins is regulated by two major pathways: the classical or canonical NF-κB activation pathway [67] and the non-canonical NF-κB activation pathway [67]. One of the most documented functions of NF-κB is its ability to promote cell survival through the induction of target genes (Figure 3), the products of which inhibit the apoptotic machinery in normal and malignant cells

Figure 3



Summary of some of the key processes regulated in the cytoplasm, at the mitochondria, in the nucleus, or in the cytosol by the PI3K/Akt pathway in controlling apoptosis. The positive events controlled either directly or indirectly by PI3K/Akt are indicated by arrows, whereas blocked lines represent events that have inhibitory effects. Bad, Bcl-2/Bcl-X_L-associated death domain protein; Bax, Bcl-2-associated X protein; Bcl-X_L, Bcl-2-related gene, long form; Bim, Bcl-2-interacting mediator of cell death; FasL, Fas ligand; IAP, inhibitor of apoptosis; IκB, inhibitor of nuclear factor-kappa-B; IKK, IκB (inhibitor of nuclear factor-kappa-B) kinase; Mcl-1, myeloid cell leukemia 1; Mdm2, murine double minute; NF-κB, nuclear factor-kappa-B; p53, 53 kDa protein; PDK-1, phosphoinositide-dependent protein kinase 1; PI3K, phosphoinositide 3-kinase; PKB/Akt, protein kinase B.

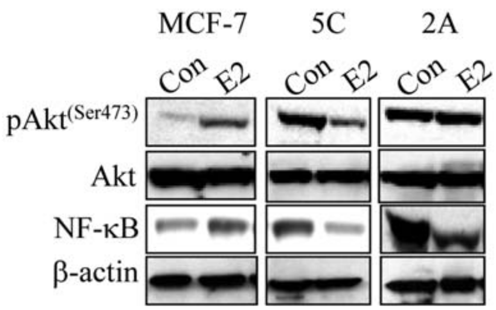
[68]. Indeed, overall reduction in NF-κB activity is associated with an increased apoptotic index in many cell types [68]. Furthermore, NF-κB activation has been shown to inhibit p53-dependent apoptosis following expression of the oncogene AP12/MALT1 [69]. Thus, blocking this signaling pathway might be a promising option to improve the efficacy of conventional anti-cancer therapies.

Several studies have shown that E2 can inhibit the activity of NF-κB and thereby increase apoptosis. For example, Osipo and colleagues [35] reported that, in tamoxifen-resistant MCF-7 tumors, E2 treatment almost completely down-regulated the level of the NF-κB p65 subunit protein, which correlated with the anti-proliferative and pro-apoptotic effects of E2 in this model system. These investigators also reported that cyclooxygenase 2 (COX-2), an NF-κB-responsive gene, was markedly reduced in E2-treated tamoxifen-stimulated MCF-7 tumors [35]. They concluded from this finding that E2-induced apoptosis and tumor regression in tamoxifen-resistant MCF-7 tumors occurred, in part, through suppression of the pro-survival/anti-apoptotic factor NF-κB. It should be noted that NF-κB expression is also markedly elevated in raloxifene-resistant MCF-7 breast cancer cells [49] and LTED breast cancer cells (Figure 4) and its downregulation by E2 is associated with the suppression of proliferation and the induction of apoptosis [31,32,70].

Glutathione suppression and estrogen-induced apoptosis

Previous studies have reported that, apart from its action on the mitochondria, Bcl-2 functions as an anti-oxidant to block apoptosis and that Bcl-2 protein levels and glutathione (GSH) intracellular concentration are coordinately regulated, with a decrease in either favoring cell death [71]. It is

Figure 4



E2 (17β-estradiol) regulation of survival pathways in wild-type MCF-7 cells and long-term estrogen-deprived MCF-7:5C and MCF-7:2A breast cancer cells. Cells were treated with 1 nM E2 for 72 hours, and protein lysates were analyzed by Western blot for p-Akt, Akt, and nuclear factor-kappa-B (NF-κB). β-actin was used as a loading control.

believed that one mechanism by which Bcl-2 may function as an anti-oxidant is through upregulation of GSH, leading to rapid detoxification of reactive oxygen species and inhibition of free radical-mediated mitochondrial damage. Bcl-2 also has the ability to shift the entire cellular redox potential to a more reduced state which is independent of its effect on GSH levels [72].

GSH is a water-soluble tripeptide composed of glutamine, cysteine, and glycine. It is the most abundant intracellular small-molecule thiol present in mammalian cells, and it serves as a potent intracellular anti-oxidant, protecting cells from toxins such as free radicals [73]. Changes in GSH homeostasis have been implicated in the etiology and progression of a variety of human diseases, including breast cancer [74], and studies have shown that elevated levels of GSH prevent apoptotic cell death whereas depletion of GSH facilitates apoptosis [75]. L-buthionine sulfoximine (BSO) is a specific γ-glutamylcysteine synthetase inhibitor that blocks the rate-limiting step of GSH biosynthesis and, in doing so, depletes the intracellular GSH pool in both cultured cells and whole animals [73].

Recently, we reported that GSH participates in retarding apoptosis in anti-hormone-resistant LTED MCF-7:2A human breast cancer cells and that depletion of this molecule by BSO, a potent inhibitor of GSH biosynthesis, sensitized these resistant cells to E2-induced apoptosis [34]. GSH levels were elevated approximately 60% in MCF-7:2A cells compared with wild-type MCF-7 cells and these cells failed to undergo apoptosis following 1 week of E2 treatment. In the presence of BSO (100 μM), however, 1 nM E2 caused a dramatic increase in apoptosis which was observed as early as 48 hours, with maximum induction observed at day 7. The apoptotic effect of E2 plus BSO in MCF-7:2A cells was associated with a marked decreased in Bcl-2 and phosphorylated Bcl-2 protein levels, mitochondrial membrane disruption and cytochrome *c* release, caspase 7 activation, and PARP cleavage [34]. It is important to note that the concentration of BSO (100 μM) used in this study is clinically achievable without significant side effects [76]. Furthermore, early-phase clinical trials of BSO at doses resulting in both peripheral and tumor GSH depletion show that BSO can be safely administered with melphalan (L-PAM) to patients with refractory disease [77,78]. Thus, it is possible that future clinical studies of BSO infusions combined with low-dose estrogen hold the promise of improving disease control for patients with anti-hormone-resistant ER-positive metastatic breast cancer.

c-Jun N-terminal kinase signaling pathway

There is also evidence that E2 induces apoptosis by regulating the c-Jun N-terminal kinase (JNK) pathway. JNKs are a group of MAPKs that bind the NH2-terminal activation domain of the transcription factor c-jun and phosphorylate c-jun on amino acid residues Ser-63 and Ser-73 [79]. JNKs are stimulated by multiple factors, including cytokines, DNA-damaging agents, and environmental stresses, and are important in controlling programmed cell death or apoptosis. The inhibition of JNKs has been shown to enhance chemotherapy-induced inhibition of tumor cell growth, suggesting that JNKs may provide a molecular target for the treatment of cancer [79]. Recently, Altiok and colleagues [80] reported that, under low growth-stimulated conditions, high concentrations (1 μM) of E2 induced apoptosis and concomitantly increased phosphorylation of c-jun in ER-positive MCF-7 breast cancer cells but not in ER-negative MDA-MB 231 cells, thus suggesting an ER-mediated event. Interestingly, when the JNK signaling pathway was disrupted by the JNK inhibitor SP600125, the ability of E2 to inhibit the growth of MCF-7 cells and to induce apoptosis was completely blocked. More recently, we reported that JNK activation (as measured by the increased levels of phospho-JNK1/2 and the JNK substrate phospho-c-Jun) was increased by low-dose E2 in the presence of BSO in anti-hormone-resistant MCF-7:2A cells [34]. While the exact mechanism by which JNK promotes apoptosis is not currently known, the phosphorylation of transcription factors such as c-jun and p53, as well as pro- and anti-apoptotic Bcl-2 family members, has been suggested to be of importance [81]. The treatment with BSO plus E2 markedly increased phosphorylated c-jun in MCF-7:2A cells and decreased phosphorylated Bcl-2 in these cells. These findings thus suggest that BSO plus E2 might mediate their apoptotic effect, in part, through activation of JNK.

Clinical exploitation of estrogen-induced apoptosis

Laboratory studies uniformly demonstrate that low concentrations of estrogen can cause apoptotic tumor cell death following profound estrogen deprivation with anti-hormones. The question that now needs to be answered is how can this new laboratory knowledge be translated into patient care?

Recently, Ellis and colleagues [42] reported that low-dose E2 (6 mg daily: 2 mg three times a day) produced a 25% response rate for patients with ER-positive AI-resistant advanced breast cancer. This number is slightly lower than the 31% objective response rate reported by Lonning and colleagues [40] with DES (5 mg three times a day) in post-menopausal women heavily pre-treated with endocrine therapy. The Lonning study [40] recruited patients with advanced breast cancer who were previously treated with exhaustive endocrine therapy. Of the 32 patients enrolled, four patients obtained complete response (CR) and six patients obtained partial response. In contrast, the Ellis study [42] recruited patients who were treated with an AI with 24 or more weeks of progression-free survival or who had a relapse after 2 or more years of adjuvant AI. Interestingly, there were no CRs in the Ellis study [42]. Clinical observations suggest that the duration of the post-menopausal period is one of the crucial factors affecting the success of low-dose estrogen therapy. In other words, the longer the estrogen deprivation period, the higher the likelihood of a response to low-dose estrogen. The fact that there were four CRs in the Lonning study [40] but none in the Ellis study [42] suggests the need for extensive estrogen blockade or withdrawal to get the best effects from low-dose estrogen.

Estrogen and bone remodeling

In addition to its role in female sexual development and reproductive physiology, estrogen plays a key role in bone cell metabolism. Estrogen contributes to the strength of a woman's skeleton by maintaining bone density. Bone is a dynamic tissue that is constantly being reshaped by osteoblasts, which build bone, and osteoclasts, which resorb bone [82]. This dynamic process is called remodeling. Osteoblasts are derived from pleiotropic mesenchymal stem cells in the bone marrow, whereas osteoclasts are multinuclear macrophage-like cells derived from hematopoietic stem cells also in the bone marrow. Bone resorption and deposition are tightly coupled, and their balance defines both bone mass as well as quality. The regulation of bone remodeling is complex; however, estrogen is thought to play a key role in this process [82]. Estrogen inhibits bone remodeling and bone resorption and enhances bone formation. Conversely, loss of estrogen, due to menopause or surgical oophorectomy, leads to an increased rate of remodeling and tilts the balance between bone resorption and formation in favor of the former [83]. Estrogen deficiency in post-menopausal women frequently leads to osteoporosis, the most common skeletal disorder. The imbalance in bone turnover that is induced by estrogen deficiency in women and female rodents can be ameliorated with estrogen/progestin hormone therapy or SERMs [84].

The main effect of estrogen on bone remodeling is to decrease activation frequency and subsequently decrease the numbers of osteoclasts and osteoblasts. Its effects on osteoclasts are mainly indirect and mediated by products secreted by the osteoblast. These products include RANK-L (the ligand of the receptor activator of NF-κB), colony-stimulating factor 1 (CSF-1), and osteoprotegerin (OPG). They regulate the differentiation of osteoclast precursors to osteoclasts and then modulate the activity of the mature osteoclasts and regulate its rate of apoptosis. Estrogen also decreases the secretion of the pro-inflammatory cytokines interleukin (IL) 1, IL-6, and TNF-α by marrow monocytes, resulting in decreased production of OPG and RANK-L by the osteoblasts, thereby decreasing the rate of production of osteoclasts, their activity, and their survival [82]. There is also evidence that estrogen has direct effects on osteoclast lineage cells. It induces apoptosis of these cells and it suppresses RANK-L-induced osteoclast differentiation by blocking RANK-L/macrophage colony-stimulating factor (M-CSF)-induced AP-1-dependent transcription through a reduction of c-jun activity [85]. Moreover, estrogen has been shown to inhibit the activity of mature osteoclasts through direct, receptor-mediated mechanisms. Indeed, a recent study by Nakamura and colleagues [86] reported that estrogen, acting via the ER-α, induced apoptosis in osteoclasts through activation of the Fas/FasL system and that this leads to suppression of bone resorption through truncating the short life span of differentiated osteoclasts.

Future perspective

Estrogen is a potent mitogen that stimulates cell proliferation and prevents cell death in breast cancer cells through activation of the ER. Paradoxically, estrogen is also capable of inducing tumor regression of hormone-dependent breast cancer in post-menopausal women who have been treated exhaustively with anti-hormones. Pre-clinical studies suggest that the evolution of anti-hormone resistance over years of therapy reconfigures the survival mechanism of the breast cancer cell so that estrogen no longer functions as a survival factor but as a death signal. It is this reconfiguration that helps to explain the 'two faces' of estrogen: the ability to stimulate growth and to induce death. Interestingly, estrogen also induces tumor regression in raloxifene-resistant endometrial tumors (G Balaburski and VC Jordan, personal communication) and it prevents bone loss by inducing apoptosis in osteoclasts, suggesting a universal principle.

Pre-clinical data [34] clearly show that it is possible to enhance the apoptotic effect of low-dose E2 by combining it with BSO. Hence, the combination of BSO and E2 could be used to improve the efficacy of E2 as an apoptotic agent if GSH depletion is fundamental to tumor cell survival. Phase I clinical trials of BSO at doses resulting in both peripheral and tumor GSH depletion show that BSO can be safely administered to patients with refractory disease. BSO was administered intravenously twice daily either alone or together with chemotherapy to cancer patients whose disease had progressed despite multiple lines of previous chemotherapy [78].

Inhibitors of survival pathways also have the ability to enhance the apoptotic/growth-inhibitory effects of E2. Several groups have developed small-molecule inhibitors of Bcl-2 as antitumor agents [87]. These inhibitors encompass various drugs that bind the anti-apoptotic Bcl-2 family members with more or less efficacy. Oblimersen (Genasense; G3139) is an anti-Bcl-2 antisense oligonucleotide that has reached phase III clinical trials in combination therapy [88]. There are also natural inhibitors of Bcl-2, which include tea polyphenols such as catechins and theaflavins [89].

Conclusions

The discovery of a new biology of E2-induced apoptosis provides a unique signal transduction pathway to exploit in the treatment of metastatic breast cancer that has become refractory to exhaustive anti-hormone therapy. The clinical clues with the use of high-dose estrogen therapy have now been supported by a wealth of laboratory data defining apoptotic mechanisms. It is plausible to consider that the methodical evaluation of monoclonal antibodies and small-molecule tyrosine kinase inhibitors to prevent breast cancer survival could amplify the apoptotic actions of estradiol in a select group of patients. Indeed, if a study of the molecular biology of estrogen-induced apoptosis can define the mechanism precisely, then the molecules involved will become the target for a new drug group. These new drugs may be able to precipitate apoptosis in ER-negative breast tumors or indeed be used universally to treat cancer types other than breast cancer.

Competing interests

The authors declare that they have no competing interests.

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REVIEW

The role and regulation of the nuclear receptor co-activator AIB1 in breast cancer

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Abstract AIB1 (**a**mplified **i**n **b**reast cancer **1**), also called SRC-3 and NCoA-3, is a member of the p160 nuclear receptor co-activator family and is considered an important oncogene in breast cancer. Increased AIB1 levels in human breast cancer have been correlated with poor clinical prognosis. Overexpression of AIB1 in conjunction with members of the epidermal growth factor receptor (EGF/HER) tyrosine kinase family, such as HER2, is associated with resistance to tamoxifen therapy and decreased disease-free survival. A number of functional studies in cell culture and in rodents indicate that AIB1 has a pleiotropic role in breast cancer. Initially AIB1 was shown to have a role in the estrogen-dependent proliferation of breast epithelial cells. However, AIB1 also affects the growth of hormone-independent breast cancer and AIB1 levels are limiting for IGF-1-, EGF- and heregulin-stimulated biological responses in breast cancer cells and consequently the PI3 K/Akt/mTOR and other EGFR/HER2 signaling pathways are controlled by changes in AIB1 protein levels. The cellular levels and activity of AIB1 are in turn regulated at the levels of transcription, mRNA stability, post-translational modification, and by a complex control of protein half life. In particular, AIB1 activity as well as its half-life is modulated through a number of post-translational modifications including serine, threonine and tyrosine phosphorylation via kinases that are components of multiple signal transduction pathways. This review summarizes the possible mechanisms of how dysregulation of AIB1 at multiple levels can lead to the initiation and progression of breast cancer as well as its role as a predictor of response to breast cancer therapy, and as a possible therapeutic target.

Keywords AIB1 · HER2 · EGFR · Estrogen

Introduction

Since the discovery in 1997 that the *AIB1* gene is often amplified in breast cancer, there has been extensive research on the role of AIB1 in breast cancer [1]. AIB1, a member of the nuclear coactivator (NCoA-3) and p160 steroid receptor co-activator (SRC) family, which includes SRC-1 [2] and TIF-2 [3], was discovered independently by several groups, and given various names; AIB1 (amplified in breast cancer 1) [1], SRC-3 (steroid receptor co-activator-3) [4], ACTR (activator of thyroid hormone and retinoid receptor) [5], RAC-3 (receptor associated co-activator-3) [6], and TRAM-1 (thyroid hormone receptor activating molecule) [7]. The mouse homologue of AIB1 is p/CIP (p/300/CBP interacting protein) [8]. The function of AIB1 as a transcriptional coactivator has been reviewed previously [9] and only a brief overview of this function is presented here. AIB1 is a transcriptional co-activator that promotes the transcriptional activity of multiple nuclear receptors such as the estrogen receptor [1, 4, 5] and a number of other transcription factors, including E2F-1, AP-1, NFκB, and STAT6 [10–13]. Three domains common to all SRC family members are involved in protein-protein interactions; an amino-terminal basic helix-loop-helix (bHLH)/Per/Arnt/Sim (PAS) domain, an internal nuclear receptor interaction domain (RID), and a carboxyl-terminal CREB-binding protein (CBP)/p300 interaction

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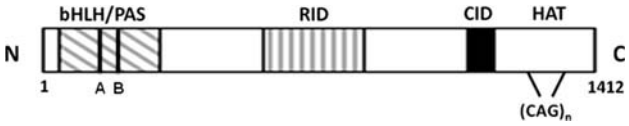

Fig. 1 Structural and functional domains of AIB1. Basic-helix-loop-helix (*bHLH*)/per-arnt-sim (*PAS*), receptor interaction domain (*RID*), CBP/p300 interaction domain (*CID*), histone acetyltranferase domain (*HAT*). A region containing multiple glutamine (*CAG*) repeats is indicated

domain (CID; Fig. 1). In addition, there is a stretch of 26–30 glutamine repeats that juxtaposes the CBP binding domain (Fig. 1) which may play a role in AIB1 function in breast cancer (see next section). After AIB1 interacts with ligand-bound nuclear receptors, via its RID, it recruits other transcriptional cofactors and the basal transcriptional machinery. Full AIB1 co-activator function also requires the recruitment of the histone acetyltransferases CBP/p300 and p/CAF [5]. Acetylation of histones by these acetyltransferases modifies chromatin structure, facilitates access of transcription factors to gene promoters and leads to enhanced gene expression [14]. AIB1 can also help transcription factors interact with other transcriptional cofactors, a role that is regulated by enzyme-dependent methylation and phosphorylation (Fig. 2). In addition to its roles in promoting transcription, AIB1 can function as a transcriptional repressor of inflammatory cytokine-encoding mRNAs [15].

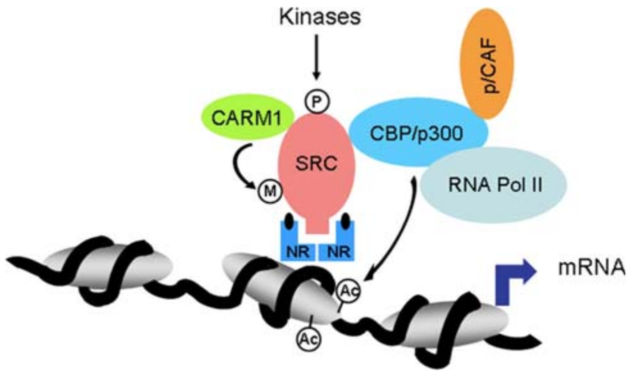

Fig. 2 Model showing a transcriptional complex of AIB1 and its methylation by the CARM1 methyltransferase and phosphorylation by multiple kinases. CARM1, coactivator-associated arginine methyltransferase 1; RNA Pol II, RNA polymerase II; CBP/p300, CREB binding-protein/E1A binding-protein p300; *NR*, nuclear receptor; *P*, phosphorylation; *Ac*, acetylation; *M*, methylation

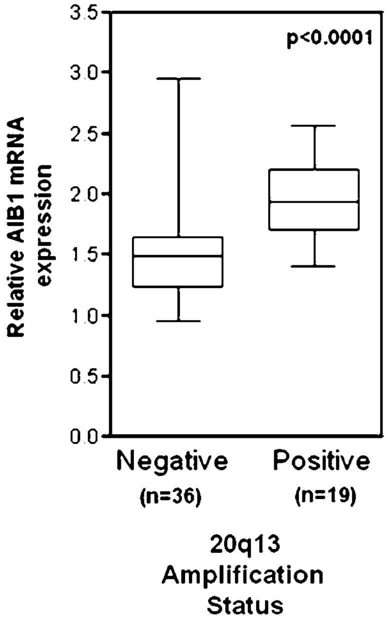

Fig. 3 AIB1 mRNA expression is significantly higher in human breast tumors with gene amplification of 20q13 (data obtained from Ginestier et al. [20] and analyzed by Oncomine 3.6 at www.oncomine.org). The median, lower and upper quartile (*box*) and extremes (*whiskers*) are shown

Evidence linking AIB1 overexpression to breast cancer risk and prognosis

Amplification of the AIB1 mRNA and protein has been shown in 2–10% of breast cancer samples [1, 16–19] and our reanalysis of Oncomine 3.6 microarray data (www.oncomine.org) from human breast cancer clinical samples from Ginestier et al. [20] shows that abnormally high AIB1 mRNA levels are associated with 20q13 amplification (Fig. 3). However, increased amounts of AIB1 mRNA have been found in 31–64% of human breast tumors [1, 21, 22] indicating that AIB1 transcript levels can be increased in breast tumors by mechanisms other than amplification of the gene. Although some studies have shown that AIB1 amplification is not associated with worse disease outcome, other studies indicate that high levels of AIB1 correlate with shorter disease-free interval [23, 24] and that AIB1 levels are higher in invasive higher grade tumors [25]. To further investigate the prognostic significance of AIB1 mRNA levels in breast cancer, we analyzed AIB1 mRNA levels using a tissue microarray, provided by CBCTR of the National Cancer Institute/NIH, that we have utilized previously for other analyzes [26] (Fig. 4a), and found that high levels of AIB1 mRNA measured by in situ hybridization (ISH) are predictive of worse outcome (Fig. 4a). In addition, we analyzed microarray data from human breast cancer samples from several studies (Fig. 5). Re-analysis of data from Richardson et al., [27] shows that AIB1 mRNA levels are significantly higher in human breast carcinomas than in normal breast tissue (Fig. 5a) and data from Farmer et al. [28] shows that AIB1 mRNA levels are higher in luminal and apocrine-type breast cancer than in basal-type breast cancer (Fig. 5b). Analysis of data from Ivshina et al. [29] shows that AIB1 mRNA level expression

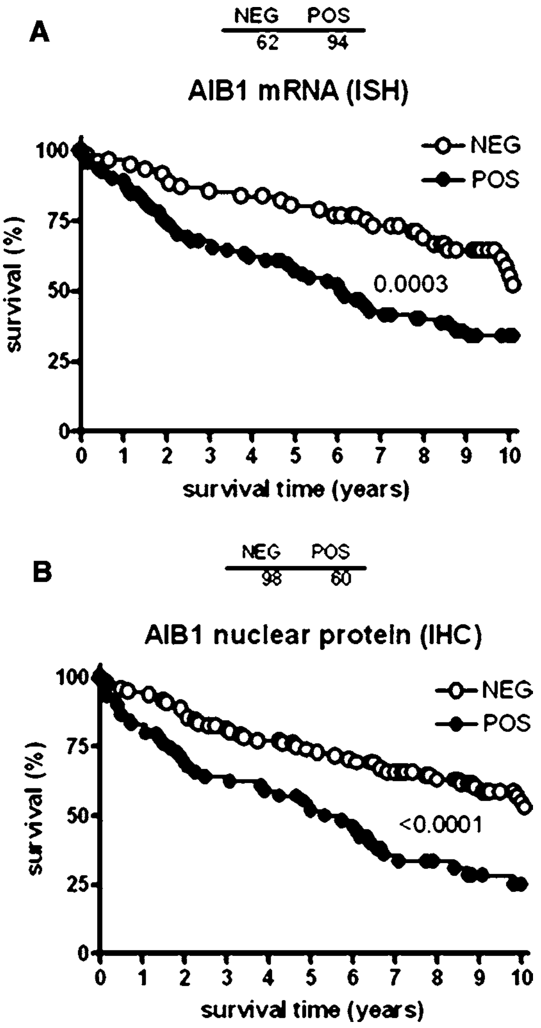

Fig. 4 Analysis of the levels of AIB1 mRNA (A) by in situ hybridization (ISH) or AIB1 protein by immunohistochemistry (IHC) on a tissue micoarray of breast cancer samples from patients with known overall survival times provided by CBCTR of the National Cancer Institute [26]

is higher in lymph node positive and high grade breast cancer (Fig. 5c, d) and analysis of a study by van de Vijver et al. [30] also reveals an association between high AIB1 mRNA levels and breast cancer recurrence (Fig. 5e). Overall, the preponderance of the published data and this analysis supports an association of high levels of AIB1 mRNA with the development of breast cancer.

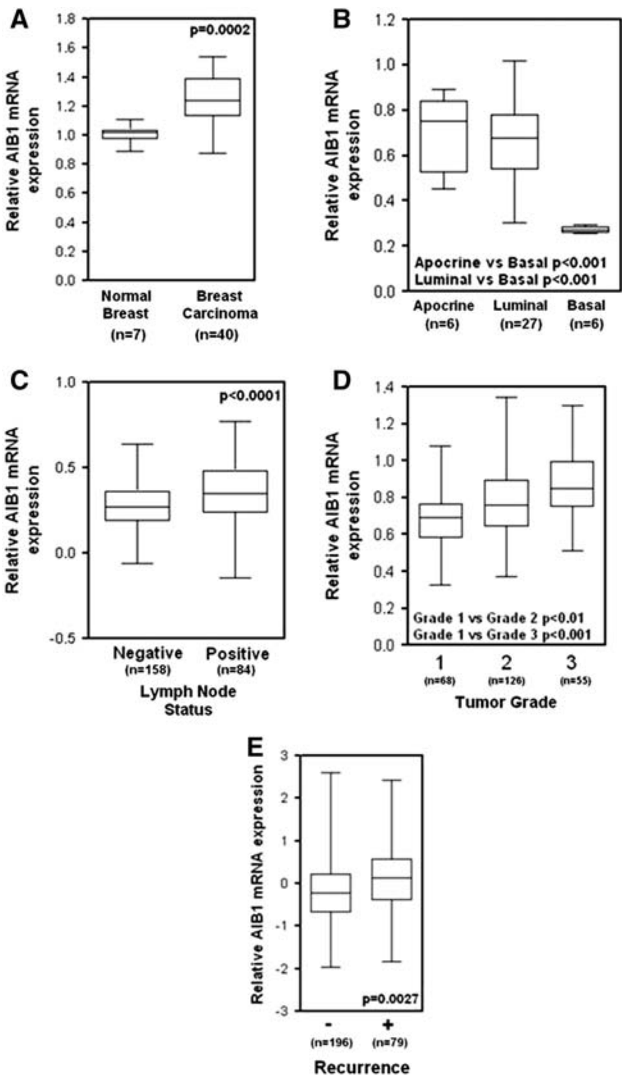

Fig. 5 AIB1 mRNA expression in breast relative to the phenotype. Data are from Oncomine 3.6 and are presented as "*box and whisker plots*" showing the median, upper and lower 25% (*boxed*) and the upper and lower extreme values (*bars*). **a** AIB1 mRNA expression is significantly higher in human breast carcinoma as compared with normal breast tissue (from Richardson et al. [27]). **b** AIB1 mRNA expression is significantly lower in basal type human breast carcinoma as compared with luminal and apocrine type breast carcinoma (from Farmer et al. [28]). **c** AIB1 mRNA expression is significantly higher in lymph node positive and **d** high grade human breast carcinoma as compared with normal breast tissue (from Ivshina et al. [29]). **e** AIB1 mRNA expression is significantly higher in early recurrence breast cancers (5 years; from van de Vijver et al. [30])

High nuclear levels of AIB1 protein have been reported in 10–16% of breast cancer patients [31]. Interestingly, cytosolic staining with AIB1 was also reported in these studies although it is not known how the relative cytoplasmic to nuclear ratio of AIB1 expression relates to disease outcome and therapeutic response. Irrespective of subcellular location of AIB1 protein, the results of some studies indicate that AIB1 mRNA levels may not always predict AIB1 protein levels. For example, List et al. [22], using imunohistochemistry (IHC), found smaller differences in nuclear AIB1 staining, between breast cancer samples and normal tissue samples, than expected on the basis of prior studies on AIB1 mRNA levels. The difference in the relative levels of AIB1 mRNA and protein in tumors could be either due to the threshold sensitivity of detection of mRNA (PCR based methods) versus protein detection by IHC or due to the complex control of AIB1 protein degradation (see "Regulation of AIB1 mRNA and protein levels" of this review). In addition, recent evidence suggests that translation of AIB1 mRNA might be dysregulated in breast cancers (see "Regulation of AIB1 mRNA and protein levels"). To study the relationship of AIB1 protein with AIB1 mRNA in tumors at various disease stages and outcome, we have compared the AIB1 nuclear protein levels measured by IHC with AIB1 mRNA levels measured by in situ hybridization in 94 breast cancer samples on a tissue microarray, provided by CBCTR of the National Cancer Institute/NIH, that we have utilized previously for other analyzes [26]. We observed that high AIB1 nuclear protein and high AIB1 mRNA levels were well correlated and predicted reduced survival rates (Fig. 4 a, b).

The AIB1 gene harbors a number of polymorphisms and some of these have been associated with reduced breast cancer risk [32]. In addition there is a polyglutamine stretch in the C-terminus of AIB1 that has variable length between 26 and 30 amino acids (Fig. 1). However, various reports show conflicting data as to the association of the repeat length with breast cancer risk. Specifically, studies have asked whether AIB1 polyglutamine length is associated with the mutation status of the tumor suppressor proteins BRCA1 and BRCA2, proteins that have roles in multiple cellular functions including cell cycle progression, DNA repair, and transcriptional regulation (reviewed in [33]). Some reports have shown that AIB1 poly-glutamine length correlates with an increased breast cancer risk in women with BRCA1 mutations [34, 35], whereas other studies did not find an increased risk for breast cancer in women with either BRCA1 or BRCA2 mutations [36, 37]. Polymorphisms in the CAG repeat region of AIB1 have also been associated with a more aggressive phenotype in ovarian cancer [38].

Evidence linking AIB1 to estrogen and progesterone effects in breast cancer

The estrogen receptor-α (ERα) and progesterone receptor (PR) status of breast cancer is an important consideration for breast cancer therapy and prognosis [39] and AIB1 is a co-activator of ERα and PR activity [40]. However, collectively, the clinical data suggest that increased AIB1 protein levels do not correlate with ERα or PR positivity [24]. One study showed that *AIB1* gene amplification in breast tumor samples correlated with increased expression of both ER and PR [41]. In another study, AIB1 mRNA overexpression in breast tumor samples was associated with loss of both ER and PR expression [21]. Our oncomine 3.6 re-analysis of data from Sotiriou et al. [42] reveals that ER-α negative breast cancer is associated with higher AIB1 mRNA levels than ERα positive breast cancer (Fig. 6). The discrepancy between these reports may be due to the differences in the role and regulation of AIB1 and the hormone receptors at different stages of breast disease. Clinical data notwithstanding, initial functional studies, that reported an association between breast cancer and elevated AIB1 mRNA/protein levels, also demonstrated that AIB1 mediates the effects of estrogen on ERα dependent gene expression [1, 22], thus suggesting a mechanism for how AIB1 influences the growth of hormone-dependent breast cancers. As predicted from this mechanistic model, depletion of AIB1 protein levels in ERα-positive MCF-7 human breast cancer cells results in decreased estrogen-stimulated proliferation and survival in culture [10, 43] and a decreased growth of MCF-7 xenografts in mice [43].

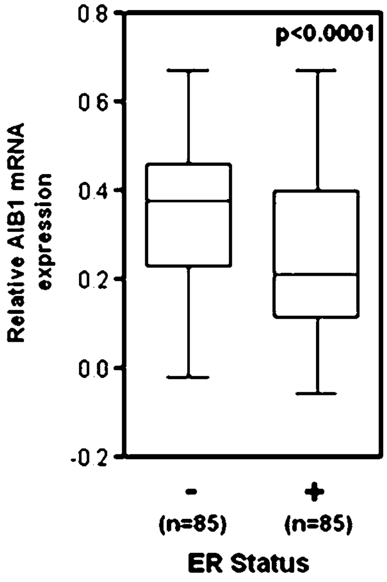

Fig. 6 AIB1 mRNA expression is significantly lower in ERα-positive human breast carcinoma as compared with normal breast tissue (from Sotiriou et al. [42] and analyzed by Oncomine 3.6)

Role of AIB1 in anti-estrogen therapy resistance

AIB1 appears to play a major role in breast cancer resistance to anti-estrogen therapy. Since the 1970s, tamoxifen has been the standard endocrine therapy for women with ER positive breast cancer. Tamoxifen is a non-steroidal estrogen receptor antagonist that competes with estrogen for binding to ERα, resulting in the inhibition of ERα-mediated transcription and estrogen-dependent cell growth [44]. Two types of resistance to tamoxifen have been recognized, intrinsic and acquired. Intrinsic resistance is associated with those 50% of ERα-positive breast cancer patients that do not respond to tamoxifen therapy [45]. In acquired resistance, patients treated with tamoxifen for long periods of time often acquire resistance to therapy.

Both types of resistances have been associated with the hormone-independent activation of ERα through cross-talk with growth factor signaling pathways [46]. Convincing clinical studies have shown that high levels of HER family member proteins have been associated with relapse after tamoxifen therapy in breast cancer patients that have high AIB1 protein expression [47–49].

While simple overexpression of AIB1 alone can increase the agonist properties of tamoxifen in breast cancer cell lines [50], the transition from hormone-dependent to hormone-independent cancer, resulting from hormone-independent ERα activation, may also be explained by increased growth factor-induced signaling through receptor tyrosine kinases such as the human epidermal growth factor receptor (HER) family members, which include HER1 (EGFR), HER2 (erbB2), and HER3 (erbB3; reviewed in [51]). Fleming et al. [52] observed that high protein expression of both HER2 and SRC-1, a p160 co-activator closely related to AIB1, is associated with resistance to tamoxifen therapy in breast cancer. Multiple studies have sought to identify the molecular mechanism causing this hormone-independent ERα activation, where tamoxifen becomes an ERα agonist in breast cancer cells with high protein levels of both AIB1 and HER2. However, an additional hypothesis linking growth factor signaling and AIB1 is that growth factor-stimulated receptor tyrosine kinase signaling may result in resistance to tamoxifen due to the enhancement of the agonist properties of tamoxifen in breast cancer cells [53]. This proposed mechanism has been corroborated by an in vitro study using ERα-positive MCF-7 breast cancer cells engineered to overexpress HER2. This study demonstrated that tamoxifen stimulates proliferation and induces ERα-dependent gene expression. Both of these effects result from the HER2-driven phosphorylation of AIB1 (via extracellular signal-regulated kinase (ERK) -1/2), which enhances AIB1 co-activator function [54]. More recently it has been shown that a balance between AIB1 and the transcriptional repressor PAX2 controls the estrogen induced expression of HER2 in breast cancer cells [55]. Tamoxifen resistance develops when AIB1 is high and PAX2 low thus inducing high HER2 expression [55].

Other endocrine therapies approved by the FDA exist for the treatment of ERα-positive breast cancer, including fulvestrant (Faslodex), a complete ERα antagonist that binds to ERα causing its subsequent degradation (reviewed in [56]). It was approved in 2004 for the treatment of postmenopausal women with metastatic breast cancer who had received prior anti-estrogen therapy. It has not yet been determined whether protein levels of AIB1 and HER2 affect the clinical outcome of breast cancer patients on fulvestrant. Aromatase inhibitors including letrozole, anastrozole, and exemestane, which block the conversion of the adrenal steroids testosterone and androstenedione into estrogen, are another FDA-approved therapeutic option for postmenopausal women with ERα-positive breast cancer (reviewed in [57]). However, breast tumors that have high protein expression of HER2 and AIB1 may also become resistant to aromatase inhibitors [58, 59].

The molecular mechanism for aromatase resistance appears to have at least some elements in common with tamoxifen resistance in that AIB1 is recruited to estrogen-dependent promoters in a hormone-independent manner in both types of drug resistance. One study showed that treatment of aromatase-expressing MCF-7 cells with androstenedione results in increased recruitement of AIB1 to the *pS2* estrogen-responsive promoter, which is inhibited by the aromatase inhibitor letrozole [59]. However, when HER2 is overexpressed in these cells, AIB1 is recruited, along with ERα, to the *pS2* promoter even in the presence of letrozole. It should also be noted that AIB1 mRNA levels are increased in MCF-7 breast cancer cells with anti-estrogens (ICI 182,780 and tamoxifen), whereas AIB1 mRNA levels are decreased by estrogen [60]. This data might suggest that during anti-estrogen therapy, AIB1 levels are increased and can contribute to resistance by enhancing hormone independent proliferative pathways. In summary, these data suggest a major role for AIB1 in anti-estrogen resistance and that it may be useful to assess the expression of HER2 and AIB1 when deciding upon the proper clinical regimen.

AIB1 in hormone -independent breast cancer

Even though AIB1 levels have been shown to be limiting for ERα-positive breast cancer growth (See "Evidence linking AIB1 overexpression to breast cancer risk and prognosis"), substantial evidence indicates that AIB1 has roles in tumorigenesis other than as a co-activator for ERα-dependent transcription. These other studies provide convincing evidence that AIB1 can stimulate the growth of breast cancer cell lines through estrogen-independent mechanisms. Overexpression of AIB1 in ERα-positive breast cancer cell lines has been shown to increase proliferation even in the presence of the ER antagonist ICI 182,780 [10]. More convincingly, overexpression of AIB1 promotes the growth of ERα-negative breast cancer cells by increasing the expression of E2F1-induced gene products such as E2F1, cyclin E, and cyclin-dependent kinase 2, all of which promote cell proliferation [10]. In one study, growth factor stimulation resulted in the release of E2F1 from retinoblastoma protein (Rb), allowing E2F1 to bind DNA and activate transcription of its target genes. AIB1 was recruited to E2F binding sites on DNA via its interaction with E2F1 and co-activated E2F-dependent transcription [10]. Thus, the ability of AIB1 to co-activate

E2F-dependent gene expression was hypothesized to be a hormone-independent mechanism by which AIB1 could promote breast tumor growth. Consistent with this hypothesis, it was subsequently shown that anchorage-independent growth of MCF10A human mammary epithelial cells, achieved by AIB1 overexpression, required AIB1 to interact with E2F1 [61].

AIB1 has also been shown to promote the growth and survival of breast cancer cells by acting as a co-activator of growth factor-stimulated activating protein 1 (AP-1), the transcription factor complex that contains Jun and Fos family members [11], and of NFκB-dependent transcription [12], which increases the expression of cell cycle and anti-apoptotic genes [62]. Inhibition of AP-1-dependent transcription in MCF-7 breast cancer cells was shown to result in inhibition of cell proliferation [63]. In breast cancer cells, NFκB has been shown to promote cell proliferation and survival [64–66]. Thus, the increased AIB1 protein levels in many human breast tumor cells can have multiple roles in tumor progression that are either dependent on, or independent of its original association with ERα-positive breast cancer. These transcriptional interactions ultimately lead to AIB1 rate limiting effects in several growth factor signaling pathways, which are discussed in the next section, that are critical to the initiation and progression of human breast cancer.

AIB1 controls different growth factor activated signaling pathways

Tumor cells depend on a diverse set of signaling pathways for growth and survival. The importance of AIB1 in the regulation of multiple growth factor activated pathways has been shown by a number of studies. The signaling response induced by insulin-like growth factor (IGF)-1, results in tyrosine phosphorylation of the IGF-1 receptor, recruitment of insulin receptor substrate (IRS) proteins, and activation of the phosphatidylinositol 3-kinase (PI3 K)/Akt/mammalian target of rapamycin (mTOR) pathway (reviewed in [67]). AIB1 was initially shown to be a factor involved in IGF-1 signaling from two independent studies involving mice with a gene deletion of AIB1 (p/CIP), both of which found reduced serum IGF-1 levels [68, 69]. On the other hand, IGF-1 serum levels were increased in transgenic mice that overexpressed AIB1 [70]. However, these effects on IGF-1 serum levels do not fully account for the regulation of the IGF-1 signaling pathway by AIB1. For example, IGF-1 receptor protein expression is increased in the mammary glands of AIB1-Δ3 (an alternatively-spliced isoform of AIB1 that lacks the N-terminal bHLH and PAS-A domains transgenic mice), but there is no change in serum IGF-1 levels [71]. In addition, cells derived from AIB1 knockout mice have an inherent deficiency in their biological response to IGF-1 stimulation; cultured hepatocytes and embryonic fibroblasts from AIB1 knockout mice are unresponsive to IGF-1-stimulated DNA synthesis [68]. Similarly, small-interfering RNA (siRNA)-mediated AIB1 knockdown resulted in decreased IGF-1-stimulated anchorage-independent proliferation of MCF-7 human breast cancer cells and IGF-I-dependent anti-anoikis [72].

In addition to regulating IGF-1 levels, AIB1 regulates the expression of other proteins involved in the IGF-1 signaling pathway. Torres-Arzayus et al. [73] have shown that inhibition of mTOR with the rapamycin analog RAD001 (Novartis) prevented mammary hyperplasia and hypertrophy originally induced by the overexpression of the *AIB1* transgene in the mouse mammary gland. In addition, RAD001 treatment inhibited the growth of tumor xenografts in mice from epithelial cells derived from AIB1-induced mammary tumors [73]. Therefore, the PI3 K/Akt/mTOR pathway has a role in AIB1-mediated tumorigenesis, but it is unclear whether this is solely due to increased IGF-1 levels or if other mechanisms are involved.

AIB1 was also identified as being involved in v-Ha-Ras-mediated tumorigenesis and transformation. AIB1 knockout mice harboring the v-Ha-Ras transgene, driven by the mouse mammary tumor virus (MMTV) promoter, were utilized to study the role of AIB1 in Ras-mediated mammary tumorigenesis [74]. The tumor latency in v-HA-Ras transgenic mice crossed with AIB1$^{-/-}$ mice increased as compared with AIB1$^{+/+}$-ras mice in both virgin and multiparous animals, and tumor development was completely abolished in ovariectomized animals [74]. Therefore, an involvement of AIB1 in Ras-dependent tumorigenesis in mammary epithelial cells was partially hormone-dependent. Loss of AIB1 decreased the incidence, growth, and metastasis of v-HA-Ras-induced mammary tumors. Interestingly, AIB1 was also shown to enhance v-Ha-Ras-induced transformation of mouse embryonic fibroblasts [75]. These studies suggest that AIB1 modulates the IGF-1 signaling pathway by regulating the expression of multiple genes encoding proteins that participate in this pathway.

There is emerging evidence that AIB1 could also be functionally involved in regulating the activity of the HER/erbB family of transmembrane receptor tyrosine kinases. This family of receptors includes EGFR/HER1/erbB1, HER2/erbB2/neu, HER3/erbB3, and HER4/erbB4, which can form either homodimers or heterodimers with each other (reviewed in [76]). A functional relationship between AIB1 and the HER/erbB family members has been shown to be clinically relevant being that HER/erbB family members are frequently activated in human breast cancers and are the target of drugs that have been successfully used for cancer therapy (reviewed in [77]). Multiple studies have demonstrated that AIB1 mRNA and protein expression in breast

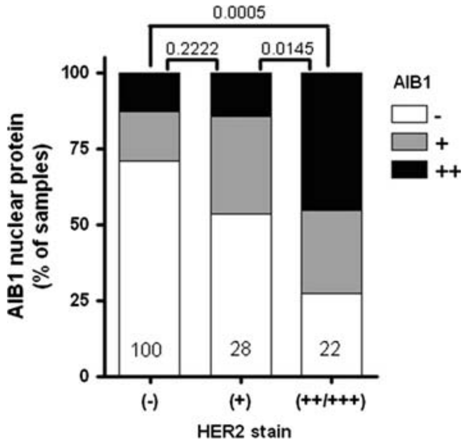

Fig. 7 High expression of AIB1 protein is associated with high HER2+ staining in a significant portion of breast cancer samples. For this study we analyzed a tissue micoarray of breast cancer samples provided by CBCTR of the National Cancer Institute [26]. HER2 status was provided by the NCI

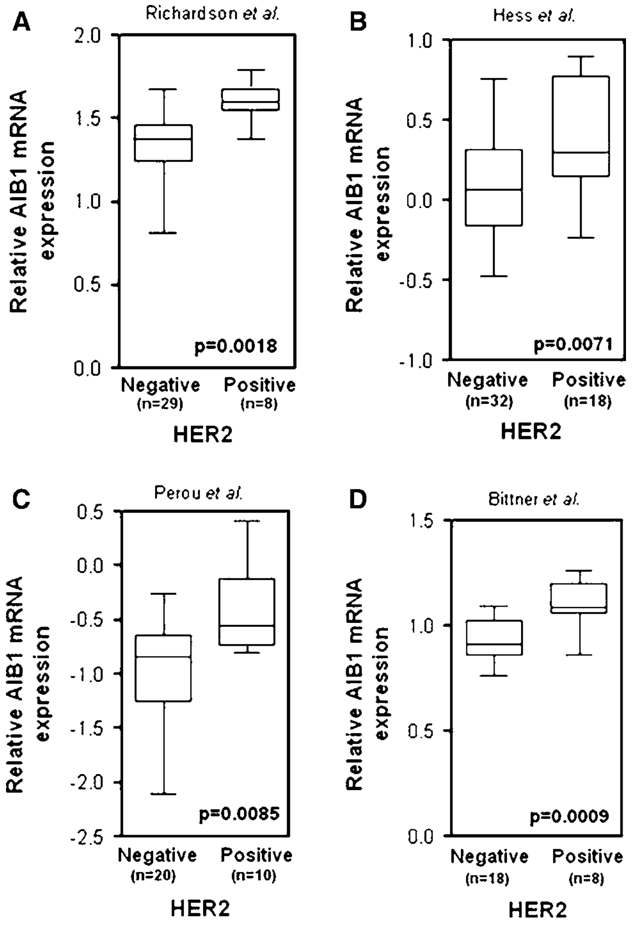

Fig. 8 AIB1 mRNA expression is significantly higher in human breast carcinoma with high HER2 expression as compared with normal breast tissue (from **a** Richardson et al. [27], **b** Hess et al. [78], **c** Perou et al. [79], and **d** Bittner et al. [80] and analyzed by Oncomine 3.6)

cancer is associated with an increase in the protein expression of HER2 [21, 47, 48]. We have also found this association in our analysis of a tissue microarray, provided by CBCTR of the National Cancer Institute/NIH, that we have utilized previously for other IHC analyzes [26] (Fig. 7). In addition, our analysis of data from four studies [27, 78–80] shows that high AIB1 mRNA expression in breast cancer clinical samples correlates with high HER2 expression (Fig. 8). A role for AIB1 in the regulation of the HER2 pathway was recently elucidated, where AIB1 was shown to be required for HER2-mediated mammary tumorigenesis in a mouse model for breast cancer through regulation of HER2 phosphorylation and signaling [81]. Generation of neu/HER2 transgenic mice with either loss of one or both copies of the AIB1 gene resulted in reduced or complete abolition of mammary tumor development, respectively. The importance of AIB1 in HER2-mediated mammary tumorigenesis may be comparable with its importance in the v-Ha-Ras transgenic mouse model where it was shown that with loss of both copies of the AIB1 gene, mammary tumors still developed albeit at a reduced rate [74].

EGFR is another family member of HER2 that has been shown to be affected by the level of AIB1 expression. This was observed in MDA-MB-231 breast cancer cells where a reduction of AIB1 levels by siRNA-mediated knockdown resulted in decreased EGF-stimulated EGFR tyrosine phosphorylation, signaling, and biological responses [82]. Our laboratory has also observed that EGF-stimulated EGFR phosphorylation was decreased in mammary epithelial cells from AIB1 knockout mice (unpublished data). These data suggest that increased AIB1 expression in breast cancer cells could enhance EGFR signaling as a result of HER2 overexpression and enable cells to be more resistant to drugs that target HER2 activity. Thus, our finding that AIB1 affects the signaling capacity of EGFR in cancer cells suggests that AIB1 may play an important role in EGFR-mediated oncogenic processes, which may have potential therapeutic applications for current therapies that are used for treating HER2-overexpressing breast cancer.

HER2 is overexpressed in 20–30% of breast cancer patients and is correlated with reduced disease-free and overall survival [83, 84]. Trastuzumab (Herceptin), a humanized monoclonal antibody directed against an extracellular region of the HER2 protein, was the first HER2-targeted therapy approved by the FDA for the treatment of HER2-overexpressing metastatic breast cancer (reviewed in [85]). Additionally, trastuzumab therapy improves disease-free and overall survival of patients with early-stage HER2-positive breast cancer in combination with chemotherapy [86–88]. Not all HER2-overexpressing breast cancers, however, respond to trastuzumab therapy or may acquire resistance during treatment [89], and breast cancers with normal HER2 levels also respond at the same rate [90]. Resistance to trastuzumab treatment has been attributed to the activation of IGF-IR signaling [91, 92], decreased p27^{kip1} expression [93–95], and the presence of truncated forms of circulating HER2 [96]. The studies reviewed here suggest that measurements of AIB1 protein levels in breast tumors may be a useful diagnostic tool for predicting treatment outcome. Since AIB1 enhances IGF-1 signaling, overexpression of AIB1 in HER2-overexpressing breast cancer cells may contribute to trastuzumab resistance through activation of IGF-IR. It will be important to determine the correlation of high AIB1 and HER2 protein expression with the clinical response to trastuzumab therapy. The ability of AIB1 to enhance the activity of multiple signal transduction pathways involved in cancer, including HER/erbB and IGF-IR, supports the possibility that AIB1 could be a target, or predictive marker for cancer therapy. There are many potential ways to target AIB1 in cancer cells, including a reduction in AIB1 expression through RNA interference-mediated knockdown of AIB1 protein levels or through inhibition of AIB1's co-activator function by disrupting its interaction with CBP/p300.

Functional studies in mice linking AIB1 to breast cancer

Mouse model studies have increased our understanding of AIB1's roles in mammary tumorigenesis. Transgenic mice expressing high levels of the human *AIB1* transgene, under the transcriptional control of the MMTV LTR, developed mammary hyperplasia and tumors of the mammary gland [70]. Interestingly, the *AIB1* transgene encoded protein was detected in other mouse tissues such as lung, pituitary, and uterus, where tumors also developed. The conclusion drawn form this study, that AIB1 protein levels may be a factor in mammary tumorigenesis, was reinforced by a study showing that mice mammary glands over-expressing AIB1, but to a lesser extent (2.5-fold vs. 7.6-fold, at the mRNA level), do not develop tumors but do develop mammary hyperplasia [97]. Another mouse model study that gave similar results, i.e. only a partial progression to mammary tumors, used a human AIB1 isoform AIB1-Δ3, and a different promoter, from cytomegalovirus (CMV) [71]. This AIB1-Δ3 isoform is a more potent transcriptional co-activator than full-length AIB1 [71]. These transgenic mice developed ductal hypertrophy of the mammary gland, along with increased proliferation of mammary epithelial cells, but did not develop mammary tumors [71]. The lack of tumor formation in the AIB1-Δ3 transgenic mice was explained by lower AIB1 protein levels than in the transgenic mice containing the entire human AIB1 protein [70]. Kuang et al. [74] showed that v-Ha-ras mammary gland tumor incidence was dramatically reduced in AIB1$^{-/-}$ mice as compared with wild-type AIB$^{+/+}$ and heterozygous AIB1$^{+/-}$ mice. Taken together, these human and animal studies provide overwhelming evidence that AIB1 overexpression plays an important role in human breast cancer. In the next sections we will examine the signaling pathways that AIB1 influences in the breast cancer cells and the potential mechanisms of regulation of AIB1 in breast cancer.

Regulation of AIB1 mRNA and protein levels

Total AIB1 protein expression is regulated at the DNA (gene amplification and transcription), RNA (translation) and protein (stability) levels. Transcription of the *AIB1* gene is controlled by regulatory sequences within the −250 to +350 base pair region of its promoter (relative to the translation initiation site), a region which contains binding sites for two transcription factors, E2F1 and Sp1 [61, 98]. Since AIB1 is a transcriptional co-activator for E2F1-dependent gene transcription, the finding of an E2F1 binding site in the *AIB1* promoter suggested that AIB1 can self-regulate [61, 98]. Interestingly, the evidence confirming this prediction of positive self-regulation, via AIB1 stimulation of E2F1-dependent transcription from the *AIB1* promoter, showed that it did not require the E2F binding site, but rather the Sp1 binding site and the binding of Sp1 to this site [98]. Total *AIB1* mRNA levels are also increased when MCF-7 breast cancer cells are treated with anti-estrogens (ICI 182,780 and tamoxifen), all-trans-retinoic acid or TGF-β and are decreased by estrogen treatments [60]. These effects are associated with increased or decreased transcription [60]. In addition, the translation of AIB1 mRNA can be regulated by endogenous microRNAs, which inhibit translation by binding to the 3′-untranslated regions of target mRNAs [99]. Specifically, the microRNA *Mir-17-5p* inhibits the translation of AIB1 mRNA, causing a decrease in AIB1 protein levels [100]. The level of this microRNA is low in breast cancer cell lines with high levels of AIB1 protein [100].

AIB1 protein levels are also regulated by proteasomal degradation pathways (reviewed in [101]). The particular pathway utilized may depend on the stimulus and/or specific post-translational modifications of AIB1. In the ubiquitin-mediated proteosome degradation pathway, ubiquitin molecules are attached to proteins by E3 ligases, resulting in their degradation by the 26S proteosome, in an ATP-dependent process [102]. For example, the E3 ubiquitin ligase E6-associated protein (E6-AP) can interact with AIB1 in MCF-7 cells, suggesting that E6-AP may target AIB1 for proteosome-mediated degradation [103]. Another E3 ligase, SCF$^{Fbw7\alpha}$, has been shown to ubiquitinate lysine residues

723 and 786 in AIB1 following GSK3-mediated phosphorylation of two AIB1 serine residues (505 and 509), leading to increased AIB1 proteosomal degradation [104]. Additionally, methylation of AIB1 by the methyltransferase CARM1 results in increased AIB1 degradation [105]. However, it is not known if the CARM1-driven degradation of AIB1 is through ubiquitin-targeted proteosomal degradation. Increased AIB1 turn-over by the 20S proteosome regulator REGγ in an ubiqutin- and ATP-independent manner occurs in MCF-7 cells [106]. REGγ also interacts with AIB1 in MCF-7 cells and modulation of REGγ levels affects AIB1 levels without affecting SRC-1 levels [106]. Additionally, atypical PKC was shown to phosphorylate AIB1 and to inhibit its proteosomal degradation, by inhibiting the association of AIB1 with the C8 subunit of the 20S core proteasome, in an estrogen-dependent manner [107]. Consistent with this, we found an association between AIB1 and the C8 subunit of the 20S core proteasome in our laboratory by using MS/MS analysis to identify proteins that co-immunoprecipitate with AIB1, from total cell lysates of MCF-7 cells (our unpublished results).

Regulation of AIB1 function/activity

The co-activator function of AIB1 is regulated by multiple cellular signaling pathways. This regulation is primarily via post-translational modifications of specific amino acids, which have distinct but often related effects; these modifications affect AIB1 co-activator function by affecting AIB1 protein-protein interactions, AIB1 sub-cellular localization, and AIB1 stability. AIB1 is modified by methylation [105, 108], sumoylation [109], and acetylation [5], however, the most studied modification of AIB1 is phosphorylation. The first evidence linking AIB1 activation to its serine and threonine phosphorylation was found in extracts from MCF-7 breast cancer cells. The extracted AIB1 could also be used as an in vitro substrate for phosphorylation by extracellular signal-regulated kinase 2 (ERK2) [110]. Subsequently, AIB1 was shown to be phosphorylated by different kinases and on multiple serine and threonine sites [75, 110]. The identity of individual AIB1 phosphorylation sites was determined by analyzing recombinant AIB1 produced in sf9 insect cells [75]. The individual phosphorylation sites included multiple serine residues (S505, S543, S857, S860, and S867) and a single threonine residue (T24) [75]. Multiple kinases such as ERK, JNK, p38MAPK, GSK3, and PKA were also shown to phosphorylate AIB1 at these sites in vitro [75]. AIB1 was shown to be phosphorylated in response to estrogen in MCF-7 cells and in response to a TNF-α in HeLa cervical carcinoma cells [12, 75]. Subsequently, estrogen-induced AIB1 phosphorylation on serine 857 was shown to be dependent on IκB kinase (IKK)-α [111]. More recently we have demonstrated that AIB1 is phosphorylated at a C-terminal tyrosine residue (Y1357) and this phosphorylation can be induced by estrogen, EGF and IGF through Abl kinase [112]. Interestingly, high levels of Y-1357 phospho-AIB1 are found in HER2/Neu induced mammary tumors [112] and in human tumors (unpublished data) suggesting that Abl kinase activation of AIB1 may play a functional role in mammary tumorigenesis. This raises the possibility that Abl kinase inhibitors such as imatinib (Gleevec) may be useful in a defined therapeutic or preventive setting in breast cancer.

The phosphorylation of AIB1 has multiple functional consequences. AIB1 phosphorylation is required for binding to other transcription cofactors such as CBP/p300 and for its ability to fully function as a transcriptional co-activator in the tumorigenic process [75, 110, 112]. AIB1 phosphorylation also regulates its proteosomal degradation. For example, glycogen synthase kinase-3 (GSK-3) was shown to phosphorylate AIB1 on serine 505 in MCF-7 cells, resulting in its proteosomal degradation [104]. GSK-3 is a serine/threonine kinase that acts downstream of Akt and is inhibited when phosphorylated by Akt [113]. Thus, AIB1 is phosphorylated on multiple serine residues and on at least one tyrosine residue by kinases that are activated by hormone, growth factor or cytokine signaling. Overall the published data suggest that AIB1's potential to participate in cross-talk between signaling pathways may be greater than that previously thought; instead of receiving cross-talk signals only from serine/threonine kinases, AIB1 might also participate in crosstalk between steroid receptors and tyrosine kinase receptors.

Conclusion

Multiple studies have demonstrated that AIB1 overexpression provides a growth advantage for breast cancer cells and promotes the development of mammary tumors in mice. AIB1 overexpression is associated with the progression of breast cancer and other epithelial cancers. Since high protein levels of AIB1 and HER2 predicts worse clinical outcome, and resistance to tamoxifen therapy, the level of expression of both of these proteins may provide an important prognostic indicator whether patients should be treated with tamoxifen or aromatase inhibitors.

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Protective Effects of Dietary Antioxidants on Proton Total-Body Irradiation-Mediated Hematopoietic Cell and Animal Survival

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Dietary antioxidants have radioprotective effects after γ-radiation exposure that limit hematopoietic cell depletion and improve animal survival. The purpose of this study was to determine whether a dietary supplement consisting of L-selenomethionine, vitamin C, vitamin E succinate, α-lipoic acid and *N*-acetyl cysteine could improve survival of mice after proton total-body irradiation (TBI). Antioxidants significantly increased 30-day survival of mice only when given after irradiation at a dose less than the calculated $LD_{50/30}$; for these data, the dose-modifying factor (DMF) was 1.6. Pretreatment of animals with antioxidants resulted in significantly higher serum total white blood cell, polymorphonuclear cell and lymphocyte cell counts at 4 h after 1 Gy but not 7.2 Gy proton TBI. Antioxidants significantly modulated plasma levels of the hematopoietic cytokines Flt-3L and TGFβ1 and increased bone marrow cell counts and spleen mass after TBI. Maintenance of the antioxidant diet resulted in improved recovery of peripheral leukocytes and platelets after sublethal and potentially lethal TBI. Taken together, oral supplementation with antioxidants appears to be an effective approach for radioprotection of hematopoietic cells and improvement of animal survival after proton TBI. © 2009 by Radiation Research Society

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INTRODUCTION

Proton radiation is an emerging treatment modality that has generated significant interest for its putative capacity to selectively increase radiation dose to the tumor while lowering the dose to non-targeted tissues (*1–6*). Furthermore, exposure of humans to high-energy protons, largely from solar particle events (SPE), is a major consideration in prolonged space travel (*7–10*). While the efficacy of proton therapy in comparison to current treatment modalities remains to be determined through randomized controlled clinical trials (*1, 2, 6*), there is a also a need to further characterize systemic responses to proton radiation *in vivo* (*7, 11*).

Total-body exposure to ionizing radiation (TBI) in humans and animals can result in multiple organ dysfunction as a consequence of toxicity to the hematopoietic, gastrointestinal or cerebrovascular systems, depending on the total dose of radiation absorbed (*12, 13*). There remains a need to develop safe and effective radioprotectors that would be required in the event of a massive radiological accident, a nuclear terrorist attack, or prolonged space travel (*12–16*). We recently reported that dietary supplementation with a mixture of antioxidants comprised of L-selenomethionine (SeM), vitamin C, vitamin E succinate, α-lipoic acid and *N*-acetyl cysteine (NAC) was effective as a preventative measure prior to total-body X irradiation or as a treatment after TBI in limiting hematopoietic cell depletion, promoting hematopoietic cell recovery and improving animal survival (*17*). We have also previously observed that TBI of mice and rats with γ rays, protons or high-atomic number and high-energy (HZE) particles resulted in oxidative stress that could be quantified by decreased serum total antioxidant capacity within 4 h of radiation exposure (*18, 19*). The observed TBI-induced decrease in serum antioxidant capacity was prevented by dietary supplementation with antioxidants (*18, 19*). Several studies have characterized the *in vivo* hematopoietic system response to proton TBI (*11, 20–22*). To our knowledge, none of the recent studies have examined hematopoietic effects of potentially lethal doses of proton TBI on 30-day survival.

The aim of the current study was to characterize the hematopoietic syndrome after TBI with 1 GeV/nucleon protons and assess the efficacy of dietary antioxidant supplementation in protecting hematopoietic cells and promoting animal survival after proton TBI.

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MATERIALS AND METHODS

Animals

Male ICR mice aged 4–5 weeks were purchased from Taconic Farms Inc. (Germantown, NY). Animals were acclimated for 7 days in the Brookhaven National Laboratory (BNL) Animal Facility. Ten animals were housed per cage with *ad libitum* access to water and food pellets. Animal care and treatment procedures were approved by the Institutional Animal Care and Use Committees of the University of Pennsylvania and BNL.

Upon acclimation, the animals were randomly assigned to the AIN-93G rodent (Control) diet or AIN-93G diet supplemented with SeM (0.06 μg/g diet), α-lipoic acid (85.7 μg/g diet), NAC (171.4 μg/g diet), sodium ascorbate (142.8 μg/g diet), and vitamin E succinate (71.4 μg/g diet); the diets were obtained from Bio-Serv (Frenchtown, NJ). The levels of SeM, vitamin E and ascorbic acid used in these studies are equivalent on a weight basis to the established maximum level of daily nutrient intake in humans that is likely to pose no risk of adverse effects. The antioxidant combination in the animal diets was formulated to provide the equivalent of 2000 mg/day, 1000 mg/day and 400 μg/day, which represent the upper limits of the established Recommended Dietary Allowances (RDAs) for vitamin C, vitamin E succinate and selenium (*23*). The control diet (AIN-93G rodent diet) contains vitamin E and selenium at levels in the animal diets that are comparable on a weight basis to the human RDA levels of these compounds. Although there is no published RDA for NAC or α-lipoic acid, these thiol supplements were formulated according to previously determined effective doses in humans, 2400 mg/day and 1200 mg/day, respectively (*24, 25*), which did not exhibit chronic toxicity.

Irradiation

Total-body irradiation of animals was performed with 1 GeV/nucleon protons [approximate linear energy transfer (LET) of 0.24 keV/μm] at dose rates ranging from 20–70 cGy/min at the NASA Space Radiation Laboratory (NSRL) at BNL. Proton irradiation was carried out within the non-stopping region (Bragg plateau) of the curve for energy deposition as a function of depth. The animals were restrained in plastic holders during radiation exposures and returned to their cages afterward. Animals were returned to the animal facility once their radiation levels were determined to be at background. Sham-irradiated animals were restrained similarly.

Peripheral Leukocyte Count Evaluations

Some animals were killed humanely 4 h after irradiation by CO_2 asphyxiation followed by cardiac puncture in a sterile fashion for peripheral complete blood cell (CBC) analyses. Blood was collected in 1.7-ml microcentrifuge tubes containing 20 units of heparin and kept at ambient temperature. A 50-μl whole blood aliquot per animal was diluted with 200 μl 5% BSA in PBS for each sample. The use of 5% BSA was a recommendation from Dr. Suresh Shelat, Director of the Pathology and Medical Laboratories at the Children's Hospital of Philadelphia, where the blood samples were analyzed. BSA in PBS solution is generally used as a diluent, especially when small volumes of blood are expected and not pooled. The BSA acts to stabilize cell membranes and creates an isosmotic medium, having no direct effect on cell counts (personal communication, Dr. Suresh Shelat). Further, compared to humans, animal leukocyte populations are known to have more frequent separation failures. In these situations, histogram and blood film review are required to identify the separation failures, verify the total count, and correct the differential. BSA is reported as a blood cell stabilizer in total blood to reduce the number of "atypical" lymphocytes and aids in the consistency of the morphological evaluation of the peripheral blood (*26*).

Samples were packaged with ice packs and shipped overnight via commercial courier for CBC analysis 16–20 h later with an ADVIA 2120 Hematology System (Bayern Diagnostic, Dublin, Ireland) at the Children's Hospital of Philadelphia.

Survival Experiment

Animals were maintained on their respective diets until they were killed except where noted. One group of irradiated animals (Control → AO) was fed the control diet until 2 h after radiation exposure, at which time the control diet was exchanged for the antioxidant-supplemented diet, which was maintained for the remainder of the experiment. Animals were evaluated twice daily after irradiation. One week after irradiation, animals were shipped via a courier service to the University of Pennsylvania quarantine facility, where they were kept for the remainder of the experiment. After radiation exposure the animals were carefully monitored for signs of general toxicity: lack of grooming, ataxia, limping, abnormal posture, paralysis, lethargy, weakness, anorexia, tremors, hunched posture, convulsions, labored respiration, bleeding, rough or strained hair coat, eye lesions, sores/wounds and red eyes (or red tears). Animals showing signs of hunched posture, labored breathing and immobility were immediately euthanized because these symptoms are associated with impending death.

Spleen and Bone Marrow Cell Isolation and Quantification

The spleen was dissected, defatted, weighed and flash frozen in liquid nitrogen. Tibiae and femurs were removed and the ends of the bones were bluntly cut. The bone marrow cavity was flushed with PBS using a sterile 22-gauge needle and resuspended to obtain a single cell suspension. Aliquots were counted using a Coulter counter.

ELISA

Blood samples were collected in 1.7-ml microcentrifuge tubes, combined with 20 units of heparin, and centrifuged at 1000 *g* for 15 min at 4°C. Plasma was then separated from the pellet, kept on ice and frozen at −80°C within 1 h of collection. The concentrations of Flt-3 ligand (Flt-3L) and TGFβ1 in heparin plasma were quantified by a sandwich enzyme immunoassay using the Quantikine ELISA kits (R&D Systems) according to the manufacturer's procedure. Briefly, thawed plasma was crosslinked to monoclonal antibodies specific for Flt-3L or TGFβ1 precoated onto a 96-well plate for 2 h at room temperature, followed by the sandwiching of analyte with enzyme-linked polyclonal antibodies specific for Flt-3L or TGFβ1 for another 2 h. Signals were acquired at 450 nm using a spectrophotometric microplate reader after 30 min of color development.

Statistical Analysis

The 30-day survival Kaplan-Meier curves were compared using a log-rank test. The CBC counts were compared between control and antioxidant treatment groups by a Student's *t* test. The statistical analyses were performed using Prism Version 2.01 (GraphPad Software, San Diego, CA) and SigmaPlot Version 10.0 (Systat Software Inc, San Jose, CA) statistical software. $P < 0.05$ was regarded as statistically significant.

RESULTS

Thirty-Day Survival of Mice after Total-Body Irradiation

The effects of dietary antioxidant supplementation on survival were determined in mice irradiated with a total-body dose of 5.9, 6.8 and 7.2 Gy 1 GeV/nucleon protons (Fig. 1). When antioxidant supplementation was initiat-

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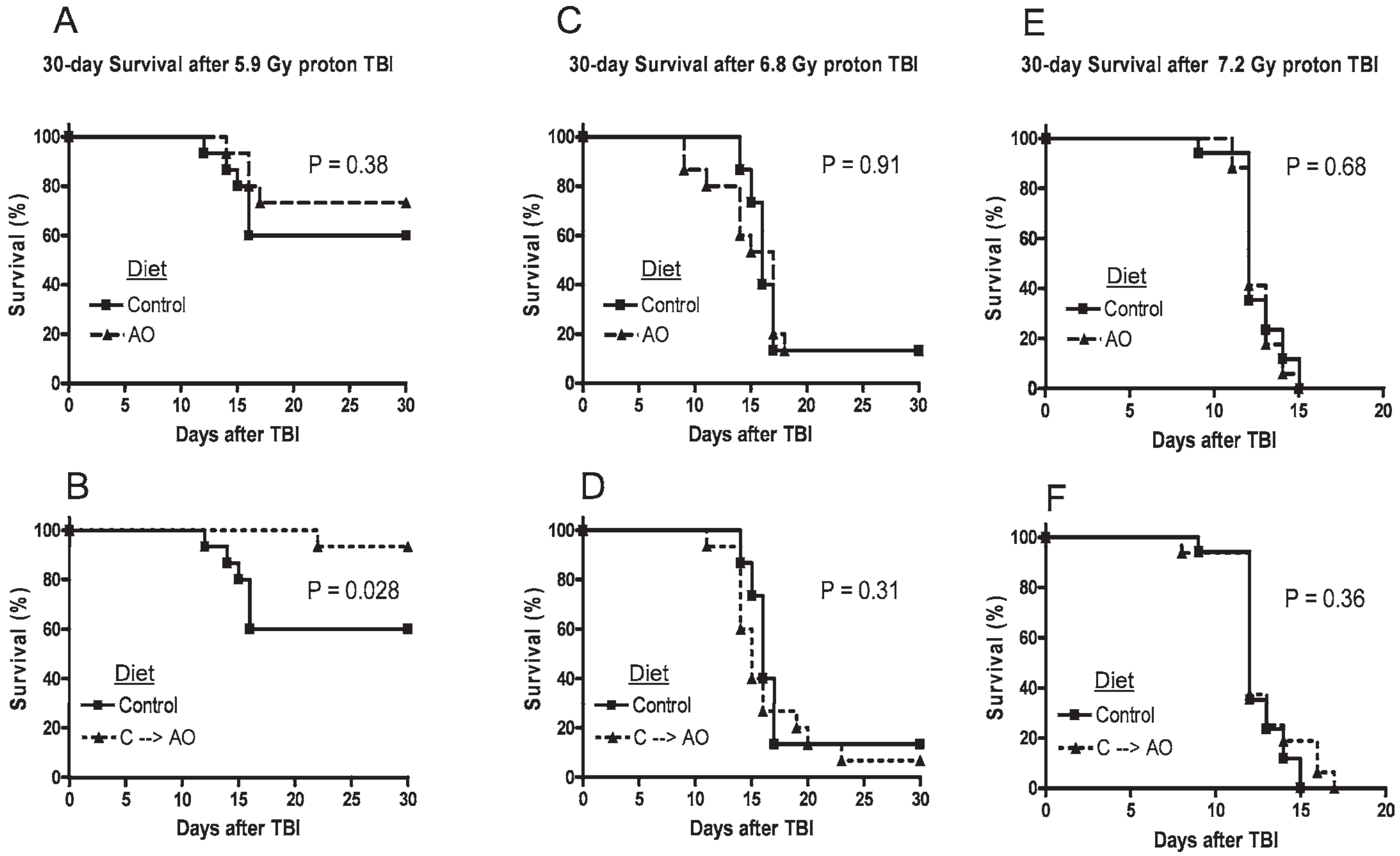

FIG. 1. Effect of antioxidants on mouse survival after TBI. Panel A: Male ICR mice were fed the control AIN-93G diet ($n = 15$) or the AIN-93G diet supplemented with antioxidants (AO, $n = 15$) for 7 days prior to 5.9 Gy TBI. The animals were maintained on their respective diets and observed for 30 days after TBI. Panel B: Survival of animals fed the control diet was compared to that of animals given the antioxidant diet 2 h after 5.9 Gy TBI and maintained on this diet for the remaining 30 days (C → AO, $n = 15$). Panels C and D: Survival of mice fed the control diet ($n = 15$) or the antioxidant-supplemented diet for 7 days prior to (AO, $n = 15$) or 2 h after (C → AO, $n = 15$) 6.8 Gy TBI. Panels E and F: Survival of mice fed the control diet ($n = 15$) or the antioxidant-supplemented diet for 7 days prior to (AO, $n = 15$) or 2 h after (C → AO, $n = 15$) 7.2 Gy TBI.

ed 7 days prior to TBI and maintained for the duration of the observation period, a small increase in survival was observed when the results were compared to those from animals fed the control diet, although this increase in survival did not reach statistical significance (Fig. 1A). However, Control → AO treatment exhibited a statistically significant survival benefit (Fig. 1B) with a hazard ratio of 0.13 (0.039–0.82). The 30-day survival rate after 5.9 Gy TBI was 60% for animals fed the control diet, 93% for the Control → AO group, and 73% for animals that received antioxidants prior to irradiation. We observed that 5.5 Gy proton TBI was sublethal in all groups (data not shown). A total-body dose of 6.8 Gy resulted in 13.3% survival that was not significantly affected by antioxidant supplementation (Fig. 1C and D, solid lines), while 7.2 Gy TBI was universally lethal in all groups (Fig. 1E and F, solid lines). There was no statistically significant difference in survival between animals that received the antioxidant supplements before or after radiation exposure (Fig. 1C–F).

Determination of $LD_{50/30}$ for 1 GeV/nucleon Proton TBI

The 30-day survivals after 5.5, 5.9, 6.8 and 7.2 Gy TBI were plotted with percentage survival as the dependent variable. Linear regression analysis demonstrated a correlation coefficient of 0.97 and a calculated $LD_{50/30}$ of 6.23 Gy for male ICR mice (Fig. 2).

Peripheral Leukocyte Counts 4 and 24 h after 1 and 7.2 Gy Proton TBI

The hematopoietic syndrome ensues within the first 24 h of total-body radiation exposure at doses as low as 1 Gy (*27*). Peripheral blood cell counts (as well as bone marrow cell depletion and spleen mass) were investigated to evaluate the effects of antioxidant supplementation on the proton TBI-induced hematopoietic syndrome at a low dose associated with the hematopoietic syndrome (1 Gy) and a lethal dose (7.2 Gy).

Whole-blood cell (WBC) counts. There was no difference in WBC counts between nonirradiated animals fed the control or antioxidant-supplemented diet

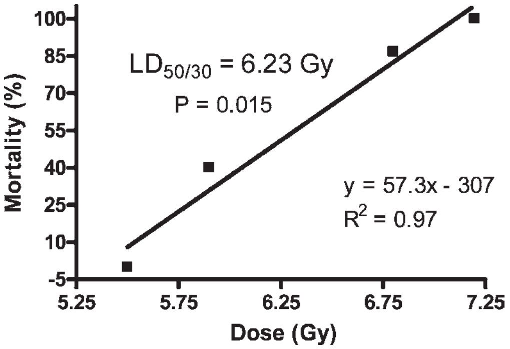

FIG. 2. Calculation of $LD_{50/30}$ for 1 GeV/nucleon protons in male ICR mice (15 per dose) fed the control AIN-93G diet for 7 days prior to TBI. The 30-day survival after TBI ranging from non-lethal (100% survival, 5.5 Gy) to universally lethal (0% survival, 7.2 Gy) was plotted as a function of radiation dose. Linear regression was used to calculate the dose equivalent of 50% 30-day animal survival.

for 7 days (Fig. 3A). At 4 and 24 h after 1 Gy and 7.2 Gy proton TBI, control animals and animals fed the antioxidant supplemented diet had significantly decreased WBC counts compared to nonirradiated animals fed the control diet (Fig. 3A). However, irradiated animals fed the diet supplemented with antioxidants exhibited significantly higher WBC counts at 4 h and 24 h after 1 Gy TBI, whereas antioxidants did not affect WBC counts after 7.2 Gy TBI in a statistically significant manner compared to irradiated animals fed the control diet (Fig. 3A).

Polymorphonuclear (PMN) cell counts. There was no difference in PMN cell counts between nonirradiated animals fed the control or antioxidant-supplemented diet for 7 days. After 1 Gy proton TBI, animals fed the control diet had significantly decreased PMN cell counts at 4 h and 24 h after radiation exposure. Supplementation with dietary antioxidants resulted in no decrease in PMN cell counts at 4 h after 1 Gy TBI (Fig. 3B). The difference in PMN cell counts at 4 h after 1 Gy proton TBI between animals fed the control and antioxidant diets was statistically significant (Fig. 3B). At 24 h after 1 Gy TBI, there were higher peripheral PMN cell counts in animals fed the antioxidant-supplemented diet compared to irradiated animals fed the control diet; this effect was of borderline statistical significance (P = 0.098). At 4 h after 7.2 Gy proton TBI, there was no change in PMN cell counts in animals fed the control or antioxidant diets compared to unirradiated animals fed the control diet (Fig. 3B). Animals exposed to 7.2 Gy proton TBI had significantly higher PMN cell counts at 4 h after radiation exposure compared to animals fed the control diet and exposed to 1 Gy TBI. Animals fed the diet supplemented with antioxidants exhibited higher PMN cell counts than animals fed the control diet at 24 h after 7.2 Gy TBI (Fig. 3B).

Lymphocyte cell counts. There was no significant difference in peripheral lymphocyte counts between nonirradiated animals fed the control or antioxidant-supplemented diets (Fig. 3C). At 4 and 24 h after 1 Gy proton TBI, there was a significant decrease in peripheral lymphocytes regardless of diet compared to unirradiated animals fed the control diet (Fig. 3C). Nevertheless, dietary supplementation with antioxidants resulted in more peripheral lymphocytes at 4 h and 24 h after 1 Gy TBI compared to irradiated animals fed the control diet (Fig. 3C). At 4 h after 7.2 Gy proton TBI, dietary antioxidant supplementation did not affect the significant decrease in peripheral lymphocytes (Fig. 3C). However, at 24 h after 7.2 Gy TBI, antioxidant-supplemented animals had higher peripheral lymphocyte counts than irradiated animals fed the control diet (Fig. 3C).

Effect of Antioxidant Dietary Supplements on Radiation-Induced Bone Marrow Cell Depletion and Spleen Mass at 24 h after TBI

Nonirradiated animals fed the control and antioxidant diets had similar bone marrow cell counts (Fig. 4A). At 24 h after 1 Gy and 7.2 Gy proton TBI, there was a significant decrease in bone marrow cell counts in animals fed the control and antioxidant diets compared to nonirradiated animals fed the control diet (Fig. 4A). Dietary supplementation with antioxidants resulted in more cells in the bone marrow of animals exposed to 1 Gy and 7.2 Gy TBI compared to irradiated animals fed the control diet (Fig. 4A).

Unirradiated animals had similar spleen mass regardless of diet (Fig. 4B). At 24 h after 1 Gy and 7.2 Gy proton TBI, there was a significant decrease in spleen mass in animals fed the control diet compared to animals fed the antioxidant-supplemented diet (Fig. 4B). Supplementation with antioxidants resulted in significantly higher spleen mass at 24 h after 1 Gy and 7.2 Gy TBI compared to irradiated animals fed the control diet (Fig. 4B).

Effect of Antioxidant Dietary Supplements on Plasma Levels of Flt-3L and TGFβ1 after Proton TBI

Flt-3L blood levels has been suggested as a biomarker of radiation injury to the bone marrow (*28, 29*). There was no difference in plasma levels of Flt-3L in nonirradiated animals regardless of diet (Fig. 5A). At 4 h after 1 Gy proton TBI, there was a statistically significant increase in plasma Flt-3L levels in animals fed the control diet (Fig. 5A), whereas there was a decrease in Flt-3L levels in animals supplemented with antioxidants that was of borderline statistical significance (Fig. 5A, P = 0.069). Antioxidant supplementation resulted in significantly lower levels of plasma Flt-3L at 4 h after 1 Gy TBI (Fig. 5A). There was no change in plasma Flt-3L levels at 4 h after 7.2 Gy TBI regardless of diet (Fig. 5A) compared to unirradiated animals fed the control diet. Furthermore, there was no difference in

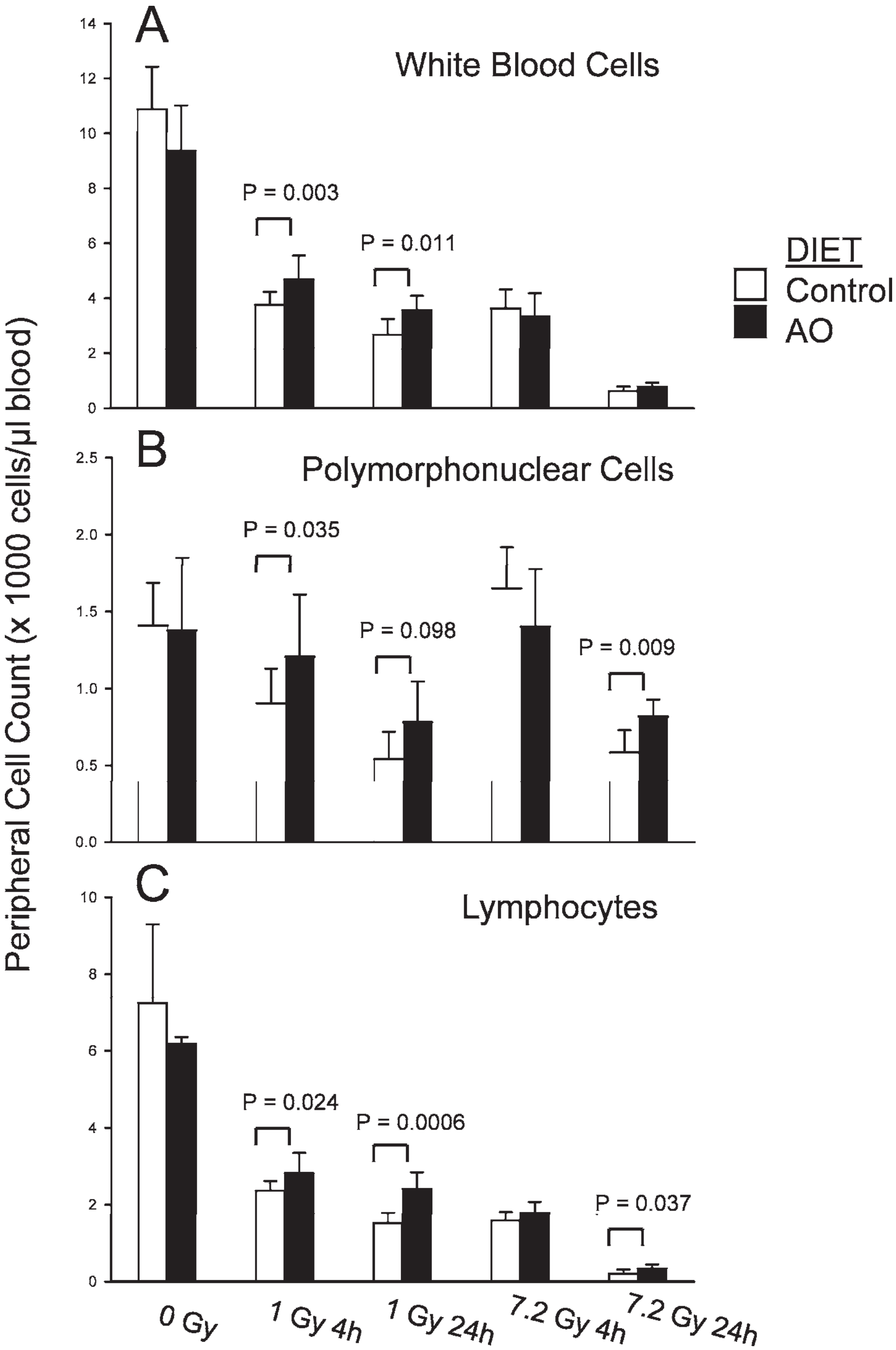

FIG. 3. Effect of prophylactic antioxidant dietary supplementation on peripheral leukocyte counts after low- and high-dose TBI. Male ICR mice were fed the control AIN-93G or the control diet supplemented with antioxidants (AO) for 7 days prior to 1 Gy or 7.2 Gy TBI and were killed at 4 or 24 h after TBI. Panel A: Total white blood cell (WBC) counts, 0 Gy control and 0 Gy AO (*n* = 4), 1 Gy control 4 h (*n* = 13), 1 Gy AO 4 h (*n* = 12), 1 Gy control 24 h (*n* = 7), 1 Gy AO 24 h (*n* = 7), 7.2 Gy control 4 h (*n* = 6), 7.2 Gy AO 4 h (*n* = 4), 7.2 Gy control 24 h (*n* = 7), and 7.2 Gy AO 24 h (*n* = 7). Panel B: PMN cell counts, 0 Gy control and 0 Gy AO (*n* = 6), 1 Gy control 4 h (*n* = 13), 1 Gy AO 4 h (*n* = 9), 1 Gy control 24 h and 1 Gy AO 24 h (*n* = 5), 7.2 Gy control 4 h (*n* = 4), 7.2 Gy AO 4 h (*n* = 4), 7.2 Gy control 24 h (*n* = 6), and 7.2 Gy AO 24 h (*n* = 6). Panel C: Lymphocyte counts, 0 Gy control and 0 Gy AO (*n* = 3–4), 1 Gy control 4 h (*n* = 10), 1 Gy AO 4 h (*n* = 10), 1 Gy control 24 h (*n* = 7), 1 Gy AO 24 h (*n* = 7), 7.2 Gy control 4 h (*n* = 5), 7.2 Gy AO 4 h (*n* = 5), 7.2 Gy control 24 h (*n* = 8), and 7.2 Gy AO 24 h (*n* = 7). Each bar represents mean ± SD.

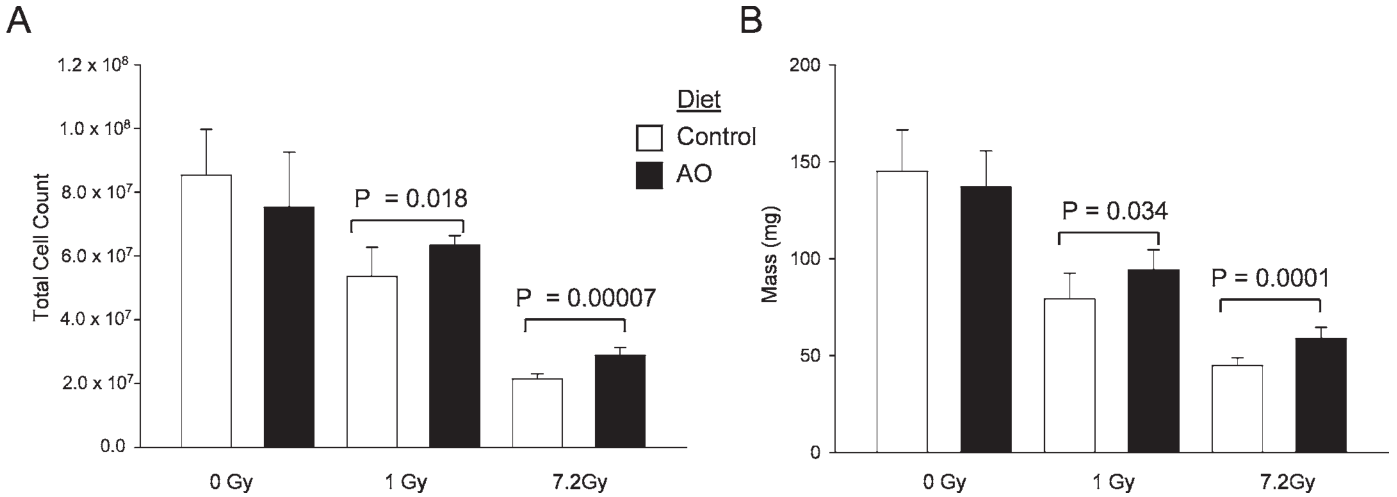

FIG. 4. Effect of dietary antioxidant (AO) supplementation on bone marrow cell depletion and spleen mass 24 h after TBI. Male ICR mice were fed the control AIN-93G or the control diet supplemented with antioxidants (AO) for 7 days prior to 1 Gy or 7.2 Gy TBI and were killed at 24 h after TBI. Panel A: Twenty-four hours after TBI, animals were killed, both femurs and tibiae were flushed with PBS, and cell counts were determined with a Coulter Counter. Each group represents $n = 5–7$. Panel B: Twenty-four hours after TBI, animals were killed, and the spleens were harvested, defatted and weighed. Each group represents $n = 7$. Each bar represents mean ± SD.

Flt-3L levels at 4 h after 7.2 Gy TBI between animals fed the control or antioxidant diet (Fig. 5A).

At 24 h after 1 Gy proton TBI, there was a significant increase in plasma Flt-3L in animals fed the control (Fig. 5A) and antioxidant (Fig. 5A) diets compared to nonirradiated animals fed the control diet. Animals fed the control diet and exposed to 1 Gy TBI had significantly higher plasma Flt-3L levels at 24 h after exposure compared to similarly irradiated animals whose diets were supplemented with antioxidants (Fig. 5A). Dietary supplementation with antioxidants did not affect the significant increase in plasma Flt-3L levels at 24 h after exposure to 7.2 Gy TBI (Fig. 5A).

TGFβ1 is one of the few negative regulators of hematopoiesis (*30–32*). Nonirradiated animals had similar levels of plasma TGFβ1 regardless of diet (Fig. 5B). At 4 h after 1 Gy proton TBI, there was a significant increase in plasma TGFβ1 levels in animals fed the control diet compared to nonirradiated animals fed the same diet (Fig. 5B). Dietary antioxidant supplementation completely inhibited the increase in plasma TGFβ1 at 4 h after 1 Gy TBI (Fig. 5B). At 4 h after 7.2 Gy TBI, there was a significant decrease in plasma TGFβ1 in animals fed the control diet compared to nonirradiated animals fed the control diet (Fig. 5B). Dietary antioxidant supplementation completely inhib-

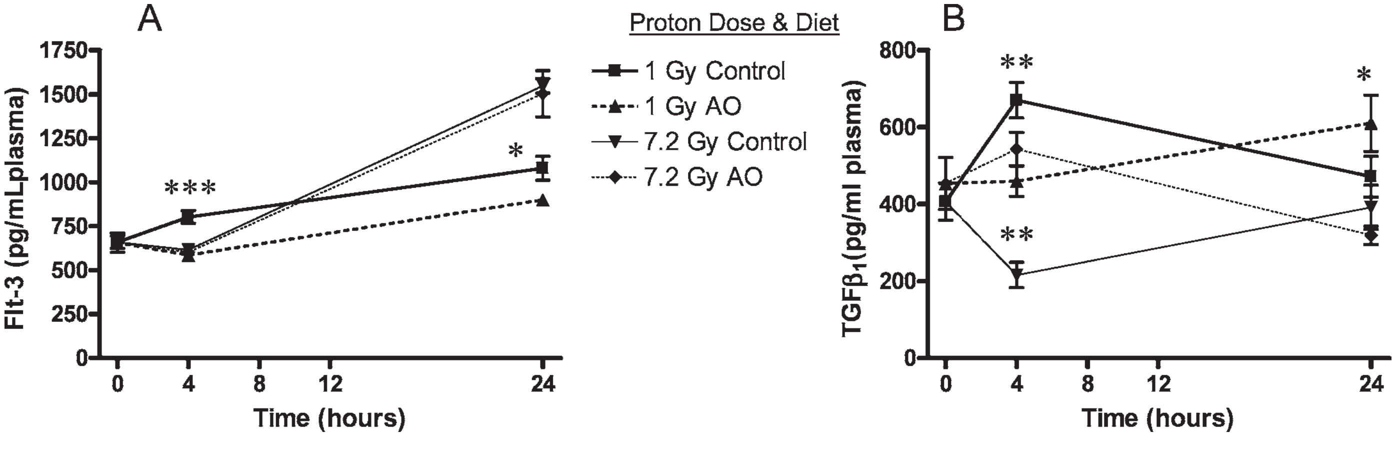

FIG. 5. Effect of prophylactic dietary antioxidant supplementation on plasma levels of Flt-3L and TGFβ1 after TBI. Male ICR mice were fed the control AIN-93G diet or the control diet supplemented with antioxidant (AO) for 7 days prior to 1 Gy or 7.2 days prior to 1 Gy or 7.2 Gy TBI and were killed 4 and 24 H after TBI; plasma was separated from peripheral blood and stored at −70°C until further analysis. Panel A: Flt-3L: 0 Gy control and AO ($n = 5–7$), 1 Gy control and AO 4 h ($n = 6–8$), 1 Gy control and AO 24 h ($n = 6–8$), 7.2 Gy control and AO 4 h ($n = 8$), 7. 2 Gy control and AO 24 h ($n = 4$). Data are means ± SD. ***$P = 0.00008$ between 1 Gy control and 1 Gy AO at 4 h. *$P = 0.018$ between 1 Gy control and 1 Gy AO at 24 h. Panel B: TGFβ1: 0 Gy control and AO ($n = 6–8$), 1 Gy control and AO 4 h ($n = 6–8$), 1 Gy control and AO 24 h ($n = 6$), 7.2 Gy control and AO 4 h ($n = 7$), 7. 2 Gy control and AO 24 h ($n = 3$). Data are means ± SD with significant difference accepted as $P < 0.05$ by Student's *t* test. **$P = 0.0013$ between 0 Gy control and 1 Gy control at 4 h. **$P = 0.0074$ between 0 Gy control and 7.2 Gy control at 4 h. *$P = 0.031$ between 0 Gy control and 1 Gy AO at 24 h. Data are means ± SD.

TABLE 1
Peripheral Blood Counts in Moribund Animals after 7.2 Gy TBI

Diet	WBC ($\times 10^3$ cells/μl)	Lymphocytes ($\times 10^3$ cells/μl)	PMN ($\times 10^3$ cells/μl)	Hct (%)	Hemoglobin (g/dl)	Platelets ($\times 10^3$/μl)
Control ($n = 6$)	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.0	8.6 ± 0.6	1.8 ± 0.3	35 ± 13
Antioxidants ($n = 3$)	0.3 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	10.7 ± 1.4	2.7 ± 0.6	45 ± 26
C → AO ($n = 4$)	0.3 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	8.7 ± 2.3	2.3 ± 0.6	58 ± 25

Note. The data are presented means ± SD.

ited the decrease in plasma TGFβ1 at 4 h after 7.2 Gy TBI exposure (Fig. 5B).

Whereas there was no longer a significant difference in plasma TGFβ1 levels at 24 h after 1 Gy TBI between animals fed the control diet and nonirradiated animals fed the same diet (Fig. 5B), 1 Gy TBI animals supplemented with antioxidants were found to have significantly higher levels of TGFβ1 compared to nonirradiated animals fed the control diet (Fig. 5B). However, the difference in plasma TGFβ1 at 24 h after 1 Gy TBI between animals fed the control and antioxidant diet did not reach statistical significance (Fig. 5B).

At 24 h after 7.2 Gy proton TBI, there was no difference in plasma TGFβ1 between irradiated animals fed the control or antioxidant diet and nonirradiated animals fed the control diet (Fig. 5B). Likewise there was no difference in plasma TGFβ1 levels between animals fed the control or antioxidant diet at 24 h after 7.2 Gy TBI (Fig. 5B).

Peripheral Leukocyte Counts in Moribund Lethally Irradiated Animals

At the time of euthanasia, 10–15 days after 7.2 Gy TBI, moribund animals were found to have a severe pancytopenia that was not affected by dietary antioxidants (Table 1).

Recovery of Peripheral Leukocyte Counts in Animals Exposed to a Sublethal Dose of Radiation

At 4 weeks after 5.5 Gy proton TBI, there remained a 52% decrease in lymphocytes, a 220% increase in PMN cells, and a 32% decrease in platelets in animals fed the control diet compared to age-matched nonirradiated animals fed the same diet (Table 2). Dietary antioxidant supplementation prior to irradiation and maintenance of this diet for 4 weeks after TBI did not affect recovery of peripheral leukocytes or platelets at 1 month after TBI (Table 2).

At 9 weeks after 5.5 Gy proton TBI, the peripheral total leukocyte, lymphocyte, PMN and platelet cell counts of animals supplemented with dietary antioxidants completely recovered to the levels observed in nonirradiated animals fed the control or antioxidant diets (Table 2). However, at 9 weeks after 5.5 Gy TBI, animals fed the control diet had significantly fewer peripheral total leukocyte and PMN cells compared to nonirradiated age-matched animals fed the control diet (Table 2). The lymphopenia observed immediately after TBI and at 4 weeks after irradiation completely resolved by 9 weeks after exposure in all treatment groups. Conversely, the granulocytosis noted in irradiated animals at 4 weeks after exposure resolved by 9 weeks as lymphocyte counts recovered.

Recovery of Peripheral Blood Counts in Animals Surviving a Potentially Lethal TBI

Eight weeks after 5.9 Gy proton TBI, the total white blood cell count in animals fed the control diet was 63% of the value of nonirradiated animals fed the same diet (Table 3). Animals supplemented with dietary antioxidants prior to TBI and maintained on this diet throughout the recovery period had 91% recovery of total white blood cell counts compared to nonirradiated animals fed the control diet (Table 3). Initiation of dietary antioxidants 2 h after TBI (C → AO) resulted in 68% recovery of peripheral total leukocytes at 8 weeks compared to nonirradiated animals fed the control diet

TABLE 2
Peripheral Blood Cell Count Recovery after 5.5 Gy TBI

Diet	WBC ($\times 10^3$ cells/μl)	Lymphocytes ($\times 10^3$ cells/μl)	PMN ($\times 10^3$ cells/μl)	Hct (%)	Hemoglobin (g/dl)	Platelets ($\times 10^3$/μl)
Control (0 Gy, $n = 4$)	9.7 ± 1.4	5.4 ± 1.1	3.0 ± 1.0	55.1 ± 1.5	17.2 ± 0.8	1522 ± 176
Antioxidants (0 Gy, $n = 4$)	10.4 ± 1.3	7.5 ± 1.1	2.1 ± 0.3	56.7 ± 2.0	17.7 ± 0.4	1502 ± 501
Control (4 weeks, $n = 4$)	10.3 ± 3.4	2.6 ± 0.8**	6.6 ± 2.1**	57.0 ± 2.9	17.4 ± 1.0	1030 ± 69**
Antioxidants (4 weeks $n = 5$)	10.4 ± 2.0	2.8 ± 0.6**	6.6 ± 1.6**	57.2 ± 1.8	17.2 ± 0.8	1130 ± 130**
Control (9 weeks, $n = 3$)	7.9 ± 0.1*	5.9 ± 0.6	1.8 ± 0.1*	52.0 ± 3.0	16.5 ± 1.1	1311 ± 143
Antioxidants (9 weeks, $n = 4$)	9.2 ± 0.8^{ns}	5.4 ± 1.0	2.9 ± 0.9^{ns}	53.0 ± 1.3	16.6 ± 0.4	1550 ± 103†

Notes. The data are means ± SD. * $P < 0.05$. ** $P < 0.01$ compared to nonirradiated animals fed the control diet. † $P < 0.05$ compared to age-matched irradiated animals fed the control diet. ^{ns} Non-significant difference from nonirradiated animals fed the control diet.

TABLE 3
Peripheral Blood Cell Count Recovery 8 Weeks after 5.9 Gy TBI

Diet	WBC (×10^3 cells/μl)	Lymphocytes (×10^3 cells/μl)	PMN (×10^3 cells/μl)	Hct (%)	Hemoglobin (g/dl)	Platelets (×10^3/μl)
Control (0 Gy, $n = 4$)	10.4 ± 1.8	7.1 ± 1.0	2.3 ± 0.7	43.5 ± 4.0	13.3 ± 1.3	1389 ± 83
Control ($n = 3$)	6.6 ± 2.0*	4.0 ± 1.0**	2.7 ± 0.7	45.8 ± 2.0	14.4 ± 1.0	906 ± 113***
Antioxidants ($n = 5$)	9.5 ± 0.5^{ns}	5.7 ± 0.7^{ns}	3.3 ± 0.9	44.5 ± 3.1	13.8 ± 1.1	1289 ± 121^{ns}‡
C → AO ($n = 6$)	7.1 ± 0.8**	4.7 ± 0.5**	2.0 ± 0.4	45.4 ± 5.5	14.4 ± 1.5	1328 ± 205^{ns}†

Notes. The data are means ± SD. * $P < 0.05$. ** $P < 0.001$ compared to nonirradiated animals fed the control diet. † $P < 0.5$ compared to age-matched irradiated animals fed the control diet. ‡ $P < 0.01$ compared to irradiated animals fed control diet. ^{ns} Non-significant difference from nonirradiated animals fed control diet.

(Table 3). Total peripheral leukocyte counts were significantly higher in animals supplemented with dietary antioxidants prior to radiation exposure compared to animals supplemented with antioxidants after TBI (Table 3) or to irradiated animals fed the control diet (Table 3).

At 8 weeks after 5.9 Gy, proton-irradiated animals fed the control diet had 56.3% (Table 3) of the peripheral lymphocyte count of nonirradiated age-matched animals fed the same diet (Table 3). Supplementation with antioxidants prior to TBI resulted in peripheral lymphocyte counts at 8 weeks that were 80% of the levels observed in nonirradiated aged-matched animals fed the control diet (Table 3). Initiation of antioxidant supplementation 2 h after TBI resulted in lymphocyte counts that were 66% of those of nonirradiated age-matched animals fed the control diet (Table 3). Lymphocyte counts were higher in animals supplemented with dietary antioxidants prior to radiation exposure compared to animals supplemented with antioxidants after TBI (Table 3) or to irradiated animals fed the control diet (Table 3).

Antioxidant supplementation, whether initiated prior to or after proton TBI, resulted in non-significant differences in platelet counts between irradiated animals and nonirradiated age-matched animals fed the control diet at 8 weeks after TBI (Table 3). However, irradiated animals fed the control diet continued to have significantly lower platelet counts than nonirradiated animals fed the same diet at 8 weeks after TBI (Table 3).

DISCUSSION

We have previously demonstrated the efficacy of dietary supplementation with a mixture of antioxidants comprised of SeM, vitamin C, vitamin E succinate, α-lipoic acid and NAC as a preventative measure prior to TBI with X rays or as a treatment after TBI in limiting hematopoietic cell depletion, promoting hemapoietic cell recovery, and improving animal survival (*17*). In the present study we aimed to study the radioprotective efficacy of the same dietary antioxidants on similar end points mediated by proton TBI. When administered as a preventative measure prior to TBI, dietary antioxidant supplementation was effective in significantly limiting radiation-induced peripheral leukopenia, neutropenia and lymphopenia at 4 and 24 h after 1 Gy, whereas the antioxidants were less effective against the hematopoietic effects of 7.2 Gy of proton TBI. Supplementation with antioxidants prior to TBI also significantly limited radiation-induced bone marrow cell depletion and the decrease in spleen mass at 24 h after exposure. Furthermore, antioxidant supplementation protected against hematopoietic syndrome-induced animal mortality in a statistically significant manner when given as a treatment after radiation exposure; survival of the irradiated animals increased from 60% in the animals fed the control diet to 93% in the animals fed with the antioxidant diet. For these data, the dose-modifying factor (DMF) for antioxidant therapy (ratio of survival of animals protected by antioxidant therapy to survival of the unprotected animals) was 1.6. The antioxidant diet was less effective in increasing survival when given in a preventative fashion before TBI. Antioxidants were effective in improving animal survival only in ICR mice exposed to a dose below the calculated $LD_{50/30}$ for 1 GeV/nucleon proton TBI. Preventative antioxidant supplementation was also associated with significant modulation of proton TBI-induced changes in plasma levels of the hematopoietic cytokines Flt-3L and TGFβ1 in a dose- and time-dependent fashion. Last, preventative supplementation with antioxidants was the most effective regimen at increasing the recovery of radiation-induced peripheral leukocyte depletion.

Several recent studies have established the short-term and long-term deleterious effects of sublethal (0.5–4 Gy) proton TBI on various hematopoietic cell parameters (*11, 20–22, 33–36*). These studies in sum elucidate the potential hematopoietic risk and harm of extended human space travel, particularly in the event of SPEs. It is worthwhile noting that the aforementioned studies used clinically relevant 250 MeV/nucleon protons. To our knowledge, none of these past studies assessed either the effect of potentially lethal doses of protons on hematopoietic cells, organs and animal survival or any countermeasures (preventative or treatment) aside from shielding (*16*). Older studies did assess the hematopoietic effects of proton TBI in dogs and primates along with

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shielding or hypoxia as countermeasures (*37–42*). Our previous *in vivo* studies with γ rays, protons or HZE particles in mice and rats suggested that dietary supplementation with antioxidants is an effective countermeasure to prevent ionizing radiation-induced decreases in plasma total antioxidant status (a marker of oxidative stress) (*18, 19*). We therefore hypothesized that dietary antioxidants would confer a protective effect against the deleterious hematopoietic effects of proton TBI *in vivo*.

The RBE of 250 MeV/nucleon protons (or lower energies) has been estimated to range from 0.9–1.25 depending on the particular end point measured and the energy of the protons used, although a general RBE of 1.1 is conventionally proposed and used (*4, 43–47*). At 70 GeV/nucleon, the proton RBE was noted to be 1.6–7.6 in Chinese hamster fibroblasts and 1.04–3.8 in lymphoid cells with single-strand DNA breaks as the end point, whereas the RBE was 1.14–1.7 for survival of Chinese hamster cells (*48*). We sought to investigate the effects of proton TBI on 30-day mortality resulting from the hematopoietic syndrome in ICR mice and to establish the RBE for this end point. The $LD_{50/30}$ for total-body exposure to X rays in ICR mice was previously estimated to be 7.55 Gy (*49*). Similarly, we found previously that 8 Gy TBI resulted in 87% mortality at 30 days in the same strain of mice (*17*). In the present study we calculated the $LD_{50/30}$ of 1 GeV/nucleon protons to be 6.23 Gy, corresponding to an RBE of 1.21 compared to the results observed for X rays. Our results are in agreement with previous *in vivo* studies and fall within the accepted 10–20% variance in RBE in the clinical setting and the conventionally accepted value of 1.1 for various proton radiation-induced biological effects (*47*).

We found that the radioprotective effect of dietary supplementation with antioxidants on animal mortality after proton TBI occurred only at a dose less than the $LD_{50/30}$, which was 5.9 Gy (equivalent to a dose of 6.5 Gy X rays, assuming an RBE of 1.1). In contrast, our previous results using X rays indicated that antioxidants significantly increased animal survival at a total-body dose of 8 Gy (greater than the $LD_{50/30}$ of 7.55 Gy). We also noticed another difference in the efficacy of dietary supplementation with antioxidants as a radioprotective countermeasure against hematopoietic injury and death induced by TBI with X rays compared to protons. Whereas antioxidants were effective at increasing animal survival when administration began at 7 days prior to or 2 h after X irradiation, dietary antioxidants were considerably more effective at increasing animal survival after proton TBI when they were administered 2 h after TBI compared to the results observed when the antioxidants were administered both before and after TBI. These data suggest that the antioxidants used in this study could be used safely as supportive therapy after proton TBI. Although the effects of the diets on animal weights were not evaluated in this study, the effects of the antioxidant diet compared to the control AIN-93G diet on animal weights and toxicity parameters were evaluated carefully in previous studies in this laboratory (*50*). In those studies, no effects on animal weight or other toxic effects were attributed to the antioxidant diet in either short-term or long-term studies involving irradiated or unirradiated animals.

The self-renewal capacity or reconstitution of hematopoietic stem cells (HSC) is dependent on ataxia telangiectasia mutated (ATM)-mediated inhibition of oxidative stress generated by p38 MAPK activity, whereas proliferation of more differentiated hematopoietic progenitor cells is less sensitive to levels of p38 MAPK-derived reactive oxygen species (ROS) (*51, 52*). Treatment of adult mice lacking the ataxia telangiectasia mutated gene ($Atm^{-/-}$) with NAC or catalase not only prevents elevation in ROS but also results in partial rescue of bone marrow failure associated with increased chronic p38 MAPK-induced ROS (*51, 52*). However, it is also known that several hematopoietic cytokines that stimulate growth, differentiation and prevention of apoptosis of progenitor cells, including granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin 3 (IL-3), steel factor (SF), thrombopoietin (TPO) and erythropoietin (Epo), cause rapid increases in ROS levels in quiescent progenitor cells via receptor-mediated signaling cascades (*53, 54*). Several antioxidants, including NAC, have been shown to abolish or diminish the receptor-mediated signaling of these hematopoietic cytokines (*53, 54*). In hematopoietic stem and progenitor cells, redox signaling mediated by NADPH oxidase and its regulatory proteins may be an important regulator of the critical balance between self-renewal and differentiation (*55*).

In the present study with proton TBI, as previously observed for total-body X irradiation, dietary antioxidants were most effective in improving animal survival when administration began 2 h after radiation exposure. Although the signaling cascades common or specific to either photons or protons are not completely known, it is evident that TBI of animals results in an inflammatory state partially mediated by oxidative stress immediately after radiation exposure that can ultimately result in animal death depending on the dose delivered. Furthermore, it remains unknown as to what extent TBI-induced oxidative stress is a necessary physiological response to promote animal survival, e.g. hematopoietic cytokine-induced receptor signaling cascades. When administered 2 h after the proton TBI, the antioxidant diet would not affect the initial oxidative stress mediated by or in response to TBI, but it could have a major effect on persistent oxidative stress induced by the radiation exposure, ultimately resulting in the most effective

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increase in animal survival after both proton and X irradiation. In our previous studies with mice and rats exposed to γ rays, protons or HZE particles, we found that dietary antioxidant supplementation prior to TBI resulted in prevention of the radiation-induced decrease in serum total antioxidant capacity (a surrogate for oxidative stress) at 4 h after exposure (*18, 19*). Although this observation is consistent with our hypothesis that TBI depletes serum antioxidant levels or other endogenous antioxidant stores, which accounts for the efficacy of dietary antioxidant supplementation, we fully acknowledge that direct measurement of antioxidants levels is necessary to confirm this mechanism of action.

In the bone marrow, total-body X irradiation at doses of 0.5–6 Gy results in a significant decrease or complete depletion of endogenous vitamin C and vitamin E levels as early as 1 h, with the nadir at 24 h after exposure (*56, 57*). These radiation-induced changes in endogenous antioxidant vitamin levels are associated with concurrent or delayed increases in markers of oxidative stress in the bone marrow including 4-hydroxynonenal, hexanal and thiobarbituric acid-reactive substances (*57*). Interestingly, sublethal TBI with 3 Gy resulted in recovery of vitamin levels in the bone marrow at 8 days after exposure, whereas there was no recovery back to normal levels after 6 Gy X irradiation (*56*). From our studies as well as those of others, antioxidant supplementation prior to or after TBI likely modifies the bone marrow response to radiation exposure.

In the current study we identified another putative means by which antioxidant supplementation affects hematopoietic cell response after TBI, which is the modulation of the hematopoietic cytokines Flt-3L and TGFβ1. Several studies have shown that after exposure to ionizing radiation, blood levels of Flt-3L are a surrogate for the extent of damage to hematopoietic progenitor cells in the bone marrow (*28, 29, 58*). Furthermore, the concentration of Flt-3L in plasma after irradiation is inversely correlated with PMN cell counts in the peripheral blood (*29*). Dietary antioxidant supplementation prior to TBI resulted in significantly lower levels of plasma Flt-3L at 4 and 24 h after 1 Gy TBI compared to levels in similarly irradiated animals fed the control diet. These results not only corroborate the protective effect of antioxidants on peripheral PMN cell counts after TBI but also suggest that preventative dietary antioxidant supplementation has a protective effect on bone marrow cell depletion after 1 Gy proton TBI. Interestingly, we observed the protective effects of antioxidants after 1 Gy TBI not only in bone marrow cell counts but also in spleen mass and peripheral PMN cell and lymphocyte counts in a similar fashion and to a similar extent. Furthermore, we observed that antioxidants did not affect the increase in plasma levels of Flt-3L after 7.2 Gy TBI. These data are consistent with the lack of difference in peripheral PMN cell and lymphocyte counts regardless of diet after 7.2 Gy TBI. The extent of peripheral leukocyte depletion after proton TBI observed in our study is consistent with results obtained from previous studies (*11, 22*). Taken together, preventative dietary antioxidant supplementation is more effective at mitigating proton TBI-induced hematopoietic cell changes at 1 Gy compared to 7.2 Gy.

Proton TBI resulted in significant changes in the plasma levels of TGFβ1 that were affected by preventative dietary antioxidant supplementation in a statistically significant manner. In animals fed the control diet, plasma TGFβ1 levels exhibited a dose-dependent response to TBI in that 1 Gy TBI resulted in significantly increased levels of the hematopoietic cytokine compared to nonirradiated control animals and 7.2 Gy resulted in significantly decreased levels at 4 h after exposure. At 24 h after TBI, TGFβ1 levels returned to the levels in nonirradiated animals in both 1-Gy and 7.2-Gy animals fed the control diet. Antioxidant supplementation resulted in an increase in TGFβ1 plasma levels at 24 h after 1 Gy compared to nonirradiated controls. Plasma levels of TGFβ1 returned to those in nonirradiated animals at 24 h after 7.2 Gy TBI regardless of diet. This suggests that antioxidant supplementation potentially abolished or delayed the endogenous TGFβ1 response. The mechanism and significance of antioxidant modulation of radiation-induced plasma TGFβ1 levels are not known. However, this is likely an important means by which antioxidants also affect bone marrow cell response or recovery after TBI. Although we did not measure plasma levels of this cytokine in our previous study with X rays, we did notice a profound effect of TBI on TGFβ1 mRNA expression in the bone marrow at 4 and 24 h after exposure (*17*). Our observation that proton TBI results in significant changes in TGFβ1 levels was not observed in the study by Kaijoka *et al.*, who compared levels of this cytokine in proton- and γ-irradiated animals (*34*). The discordance between these studies likely represents evaluation of cytokine levels at different times. Kaijoka *et al.* evaluated plasma TGFβ1 at 7 days after TBI (*34*), and we report that by 24 h the elevation of this cytokine in the circulation returns to levels observed in nonirradiated animals.

In this study we also observed that antioxidant supplementation increased peripheral leukocyte cell recovery when given prior to sublethal or potentially lethal proton TBI. The benefits of antioxidants in improving recovery of hematopoietic cells in the periphery and bone marrow were also observed in our study with X rays (*17*). Interestingly, despite a lower impact on animal survival, antioxidant supplementation before TBI resulted in the greatest improvement in hematopoietic cell recovery. These results suggest that the end point of animal survival after potentially lethal TBI is affected by many factors, including bone marrow as well as peripheral hematopoietic cell protection and recovery.

This report shows that the effects of proton TBI on hematopoietic end points, including 30-day survival, are not completely similar to the effects observed for total-body X irradiation. Some differences may be related to the higher RBE of protons compared to photons. Dietary antioxidant supplementation may be an effective countermeasure for proton-induced hematopoietic effects. However, additional studies are needed to elucidate the endogenous hematopoietic oxidative stress response to TBI and the impact and ideal timing of exogenous antioxidants on this important regulatory hematopoietic pathway.

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Review

Targeting of tamoxifen to enhance antitumour action for the treatment and prevention of breast cancer: The ‘personalised’ approach?

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ABSTRACT

Tamoxifen is a standard endocrine therapy for the treatment of steroid receptor positive breast cancer. Tamoxifen efficacy depends on the formation of clinically active metabolites 4-hydroxytamoxifen and endoxifen which have a greater affinity to the oestrogen receptor and ability to control cell proliferation as compared to the parent drug. The cytochrome P450 2D6 enzyme plays a key role in this biotransformation and lack of tamoxifen efficacy has been linked to low activity. There is now considerable mechanistic, pharmacologic and clinical pharmacogenetic evidence in support of the notion that CYP2D6 genetic variants and phenocopying effects through drug interaction by CYP2D6 inhibitors influence plasma concentrations of active tamoxifen metabolites and negatively impact tamoxifen outcome. These interrelations are particularly critical for patients with non-functional (poor metaboliser) and severely impaired (intermediate metaboliser) CYP2D6 variants, and, moreover, for patients in need of co-medication such as serotonin re-uptake inhibitors to control adverse effects such as hot flashes and other menopausal symptoms. Therefore, in the future, a personalised approach for an optimal tamoxifen benefit should consider a CYP2D6 genotype guided adjuvant endocrine treatment strategy and avoid non-adherence as well as strong CYP2D6 inhibitors such as co-medications.

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Review criteria

Apart from select historically relevant papers and review articles, literature was identified by searching the PubMed database for relevant publications written in English between December 2003 and April 2009. Search terms included ‘tamoxifen, CYP2D6 metabolism’, ‘tamoxifen outcome, CYP2D6’, and ‘tamoxifen adherence’ matched by ‘pharmacogenetics’ and/or ‘hot flashes’.

1. Introduction

Tamoxifen, a non-steroidal antioestrogen[1] (Fig. 1), is used for the treatment of all stages of breast cancer[2–4] and in the US is available to reduce the incidence of breast cancer in both pre- and postmenopausal women at elevated risk.[5–7] It is important to remember that during early clinical studies tamoxifen did not show any improvement in efficacy over standard hormonal treatments (high dose oestrogen or androgen) for

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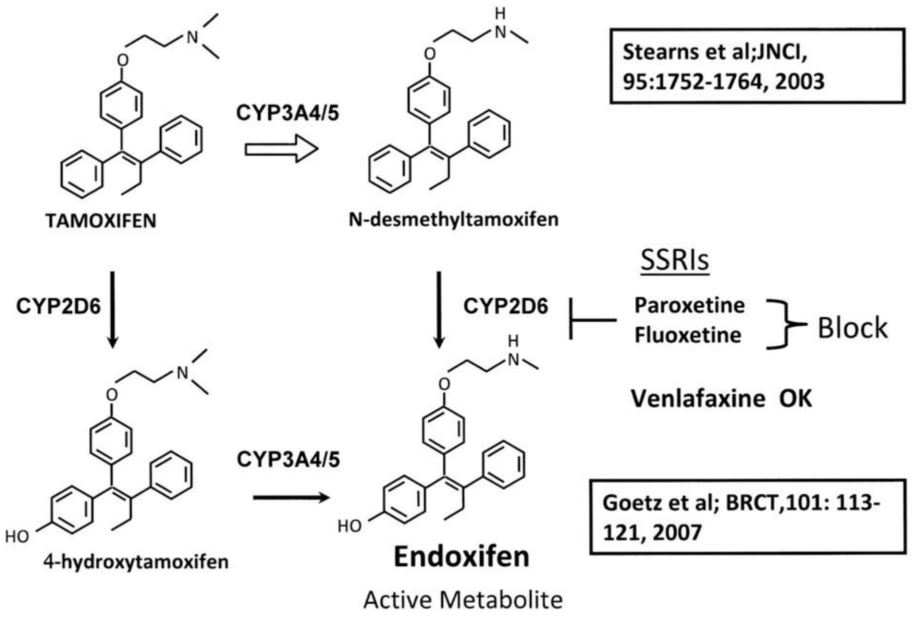

Fig. 1 – The principal metabolites of tamoxifen illustrating the route of metabolism for endoxifen via the CYP2D6 enzyme. An increase in the intensity of hot flashes and menopausal symptoms during tamoxifen therapy has prompted the widespread use of selective serotonin re-uptake inhibitors (SSRIs) to improve the quality of life. However, the SSRIs such as paroxetine and fluoxetine are also metabolised by the CYP2D6 enzyme as it can block the production of endoxifen. Venlafaxine has a low affinity for the CYP2D6 enzyme and is therefore recommended as an alternative to ameliorate menopausal symptoms.

metastatic breast cancer.[2,8] The only advantage of tamoxifen was a reduced incidence of side effects for those 30% of patients who responded for about 1 year. However, laboratory studies to target the tumour oestrogen receptor (ER)[9] employed long term adjuvant therapy[10] and considered the chemoprevention of breast cancer.[11,12] Tamoxifen was thus reinvented from an orphan drug to the 'gold standard' for the endocrine treatment of breast cancer between 1984 and 2004. The targeting of tamoxifen to block oestrogen stimulated breast tumour growth with long term (5 years) adjuvant tamoxifen therapy[13] resulted in a major improvement in patient survivorship and has contributed significantly to the reduction in national death rates from breast cancer.[14,15] The recent development of aromatase inhibitors as an effective treatment for breast cancer in postmenopausal patients[16] has improved disease-free survival and reduced the side effects of endometrial cancer and blood clots noted with tamoxifen.[17–20] However, aromatase inhibitors are not universally available in national health care systems worldwide because of significant financial constraints. Tamoxifen remains a cheap, life-saving, targeted therapy for both pre- and postmenopausal patients with breast cancer.

The application of the ER as a tumour target to treat breast cancer patients appropriately provided a valuable, but admittedly not perfect, test to increase the probability of tumour growth control during long term adjuvant treatment. Tamoxifen does not enhance either disease-free or overall survival in patients with ER negative tumours.[14,15]

At present, there are no universally accepted tumour markers to improve response rates for patients with ER positive tumours. However, emerging data on the pharmacogenomics of tamoxifen metabolism through the CYP2D6 enzyme and new knowledge of potential drug interactions with selective serotonin re-uptake inhibitors (SSRIs), to control hot flashes, provide valuable new information to aid in the selection of the appropriate long term endocrine treatment for breast cancer patients with ER positive disease.

The goal of this concise review is to describe the new understanding of the metabolic activation of tamoxifen to its putative active agent endoxifen[21–23] and consider the clinical significance of CYP2D6 polymorphisms together with phenocopying effects through drug interaction. We will summarise the actions necessary to improve the value of tamoxifen as a 'personalised targeted treatment for breast cancer'.

2. Clinical pharmacology of tamoxifen

2.1. *Tamoxifen efficacy*

Our evolving understanding of the relevance of tamoxifen metabolism for its pharmacology has recently been reviewed.[24] Nevertheless, the important pharmacological issues and conclusions will be restated to provide a scientific background for evaluating the role of the CYP2D6 enzyme and underlying genetics for the antitumour actions of tamoxifen.

Tamoxifen is a pro-drug that requires metabolic activation to 4-hydroxytamoxifen[25,26] and 4-hydroxy-N-desmethyltamoxifen (endoxifen) (Fig. 1) in order to exert its therapeutic effect.[3,4,22,23] 4-Hydroxylation of tamoxifen and its major metabolite N-desmethyltamoxifen increases the affinity for the ER,[26–28] and although both metabolites are equipotent with respect to ER binding and inhibition of 17beta-oestradiol induced cell proliferation, it is proposed that endoxifen is the principal antioestrogenic metabolite for the antitumour activity observed in breast cancer patients treated at the 20 mg daily dose of tamoxifen.[29] Endoxifen was found at more than six-fold higher concentrations in the plasma of tamoxifen treated patients as compared to 4-hydroxytamoxifen. The metabolism of interest is illustrated in Fig. 1 and the principal metabolites of interest are 4-hydroxytamoxifen and endoxifen. Both metabolites induce similar changes on global gene expression patterns, i.e. the gene array analysis of the spectrum of genes activated or suppressed by 4-hydroxytamoxifen and endoxifen in MCF-7 breast cancer cells is almost the same.[30] There are 4062 total genes either up or down regulated by oestradiol but in the presence of oestradiol and 4-hydroxytamoxifen or endoxifen, 2444 and 2390 genes were affected, respectively. Both tamoxifen metabolites showed overlapping effects on 1365 oestradiol sensitive genes and there was reasonable confirmation with selected genes by RT-PCR. The overall conclusion was that 4-hydroxytamoxifen and endoxifen are almost identical.[30] Together with the ER binding profile and the antiproliferative action of 4-hydroxytamoxifen and endoxifen in MCF-7 cells being identical,[28] but the circulating levels of endoxifen in patients being higher than that of 4-hydroxytamoxifen,[23,29] based on the Law of Mass Action, endoxifen would be anticipated to be the principal metabolite blocking the binding of oestradiol at the tumour ER.

An intriguing aspect of the investigations of the molecular pharmacology of endoxifen is the recent report that the antioestrogen targets ERα for rapid destruction in breast cancer cells.[31] The implication is that the shape of the endoxifen ERα complex is perturbed significantly for rapid proteasomal degradation. Profound structural perturbations of the ER are noted with the pure antioestrogen ICI164384[32] and the SERM GW5638[33] with both compounds causing rapid destruction of ER. In contrast, the structure of endoxifen is almost identical to the related metabolite 4-hydroxytamoxifen (Fig. 1) that causes accumulation of the ER. The structure of the 4 hydroxyl tamoxifen ER complex has been resolved.[34] Perhaps crystallisation of the endoxifen ER complex would provide insight into the actions of endoxifen at the ER.

2.2. *Tamoxifen pharmacogenomics*

2.2.1. *The role of cytochrome P450 2D6*

Numerous drug metabolising enzymes, particularly of the cytochrome P450 (CYP) iso-enzyme family, are involved in the metabolism of tamoxifen. Among these, CYP2D6 plays the dominant role in the conversion from the major, but clinically inactive, metabolite N-desmethyltamoxifen into the clinically active endoxifen (Fig. 1).[35] Together with CYP2B6, CYP2C9, CYP2C19 and CYP3A4, it is also involved in the formation of 4-hydroxytamoxifen. With CYP2D6 being at the heart of tamoxifen action, host factors, by virtue of the patients genetic makeup, must be taken into account, in addition to tumour characteristics such as ER status, in order to understand drug efficacy. This is owing to the fact that the CYP2D6 phenotype is variable and that this variability differs with respect to degree, underlying genetic variation and frequencies across ethnic groups. By way of clinical observation, the first CYP2D6 phenotypic variation (sparteine/debrisoquine polymorphism) distinct from an extensive metaboliser (EM) phenotype was identified more than 30 years ago and termed poor metaboliser (PM).[36,37] Since then, based on drug oxidation capacity, four different CYP2D6 phenotypes, namely EM, intermediate metaboliser (IM), PM, and ultrarapid metaboliser (UM), have been identified.[38–40] Their frequencies and global distributions have been investigated and extensively reviewed.[41] Although not all CYP2D6 phenotypic variations can be attributed to genetic variations, as of today, there are more than 100 known different alleles of the CYP2D6 (http://www.cypalleles.ki.se).

The PM phenotype is present in 7 to 10% of the European population with PM individuals carrying two non-functional (null) alleles leading to a loss of enzyme function. Of the numerous known null alleles the CYP2D6 *3, *4, and *5 alleles are prevalent in populations of European descent with *4 being present in 70–90% of all PMs. PM status, i.e. lack of catalytic function, can be deduced with greater than 99% certainty from the presence of two non-functional alleles and, therefore, can be accurately predicted from the patients genotype without the need to phenotype.[38,40,42,43] Ten to 15% of Europeans are IM, characterised by severely impaired CYP2D6 expression and function due to the presence of *9, *10, and *41 alleles.[39,44–46] IMs are genetically either homozygous for IM mutations or compound heterozygous for an IM allele in combination with one null allele.[45,47] The EM phenotype results from the presence of one or two alleles with normal expression level and catalytic function such as *1 and *2 and represents the most frequent CYP2D6 phenotype within the European population accounting for 60–70%. EMs can be separated into homozygous or heterozygous EMs depending on whether they carry two or one functional allele. Heterozygous EMs carrying one *1 or *2 allele in combination with an IM or PM allele have a somewhat impaired enzyme expression and function, a reason why they have been classified as ‘intermediate metabolisers’ assuming a gene dose effect. However, due to the substantial overlap both in enzyme content and activity between homozygous and heterozygous EMs, this is not correct and, therefore, the predictive value of the heterozygous EM genotype is rather poor. Importantly, the IM is a phenotype and genotype distinct from the heterozygous EM based on the presence of *9, *10, and *41 and/or non-functional alleles.[39,46] The UM phenotype is present in 10–15% of the European population and a gene duplication with up to 13 gene copies involving *1, *2, and *4 alleles has been identified as an underlying molecular mechanism.[48,49] Such an increase in enzyme activity can have profound consequences on the plasma concentrations of drug metabolites[50,51]; however, only 20–30% of the UM phenotype in the Caucasian population are accessible through genotyping and, therefore, the predictive value is rather low.[38,40,52]

While CYP2D6 tamoxifen pharmacogenomics for patients of European descent must primarily focus on PM and IM, but also include UM, the PMs play a minor role in individuals of non-European descent. Rather, within Asian populations, IMs are prevalent based on a much higher frequency of the *10 allele, i.e. 57% in Han Chinese[41] and, therefore, tamoxifen pharmacogenomics in Asia requires a focus on IM. Furthermore, North Eastern African populations would require a focus on gene duplication due to a much higher frequency e.g. 29% in Ethiopia[53] and 21% in Saudi Arabia[54] as compared to 1–5% in populations of European descent.[41,43,55]

2.2.2. *CYP2D6 genotype – tamoxifen outcome relationship*

Within recent years an increasing awareness of the CYP2D6 phenotypes and underlying genotypes[29,56] sparked a number of international clinical studies to assess retrospectively the potential value of tamoxifen pharmacogenomics for the prediction of treatment outcome of (mainly) early breast cancer. The first evidence linking CYP2D6 variants with treatment response was obtained by Goetz et al.[57] from a US prospective randomised phase III trial of postmenopausal women with primary ER positive breast cancer (North Central Cancer Treatment Group Adjuvant Breast Cancer Trial 89-30-52) investigating the effect of adding the androgen fluoxymestrone, for 1 year, to the standard 5-year adjuvant tamoxifen (20 mg/day). Patients who had received 20 mg/daily adjuvant tamoxifen ($n = 223$ of 256 eligible; mainly of European descent) were genotyped for CYP2D6 variants *4 and *6. Their genomic DNA was obtained from paraffin-embedded tissue specimens. Of the 190 patients for whom CYP2D6 (*4) genotyping was possible, 137 (72.1%) had wt/wt, 40 (21.1%) wt/*4, and 13 (6.8%) *4/*4 genotype. The concordance rate between the genotype obtained from additional buccal cells in 17 patients and the corresponding tumour tissue was 100%. After a median follow-up of 11.4 years, the CYP2D6 *4/*4 genotype was associated with poor patient outcome. CYP2D6 *4/*4 was associated with worse relapse-free ($P = 0.023$) and disease-free survival ($P = 0.012$). The genotype did not have an impact on overall survival ($P = 0.169$). The authors confirmed their findings in an extended study of 256 patients.[58]

A robust association between CYP2D6 genotypes and treatment outcome has been obtained by Schroth et al.[59] from a non-randomised retrospective cohort of ER-positive postmenopausal breast cancer cases from Germany. The study included 206 breast cancer patients treated with adjuvant tamoxifen monotherapy (standard dose) and 280 patients without tamoxifen. The comprehensive genotyping approach using constitutional DNA derived from formalin-fixed paraffin-embedded normal breast tissues included the CYP2D6 variants *4, *5, *10, and *41 to cover the vast majority of PM and IM genotypes (e.g. 95% and 90%, respectively). The analyses were aimed at the investigation of approximately 15–25% of patients expected to be carriers of PM or IM alleles and genotypes. At a median follow-up of 71 months, carriers of CYP2D6 *4, *5 *10 and *41 alleles had significantly more breast cancer recurrences, shorter relapse-free time (hazard ratio (HR) = 2.24; 95% confidence interval (CI), 1.16–4.33; $P = 0.02$), and worse event-free survival (HR = 1.89; 95% CI, 1.10–3.25; $P = 0.02$) compared to carriers of functional alleles (Fig. 2). These associations were not observed in postmenopausal ER positive patients not treated with tamoxifen. This study also included other tamoxifen metabolising genes (i.e. CYP2C19, CYP2B6, CYP2C9, and CYP3A5) and variants. Interestingly, the CYP2C19*17 (UM) allele also had a favourable effect on tamoxifen treatment outcome in that patients with a homozygous *17 genotype had significantly less breast cancer recurrences, longer relapse-free time and better event-free survival (HR = 0.45; 95% CI, 0.21–0.92; $P = 0.03$) compared to non *17 carriers.[59] Altogether, this study suggested that genotyping for CYP2D6 *4, *5, *10 and *41 can identify patients who will derive little benefit from adjuvant tamoxifen. However, EM patients, accounting for approximately 50% of all patients, are likely to benefit from tamoxifen and this benefit will be at a maximum for those with a combination of CYP2D6 functional and CYP2C19 UM alleles. The latter applies to approximately one third of all patients pointing to the relevance of tamoxifen pharmacogenomics for a large fraction of patients receiving endocrine treatment.

Supportive evidence has been provided by studies from Korea,[60] China[61] and Japan.[62] As expected for Asian populations, the CYP2D6 *10 allele significantly contributed to the overall fraction of IM genotypes and observed effects in these patient cohorts. The Korean study by Lim et al.[60] included 202 patients with either primary or metastatic breast cancer treated with 20 mg/daily tamoxifen for more than 8 weeks. Genotype frequencies were 31.6% for wt/wt, 44% for wt/*10, and 24.2% for *10/*10. Patients with *10/*10 genotype ($n = 49$) had significantly lower steady-state plasma concentrations of endoxifen and 4-OH-tamoxifen than those with other

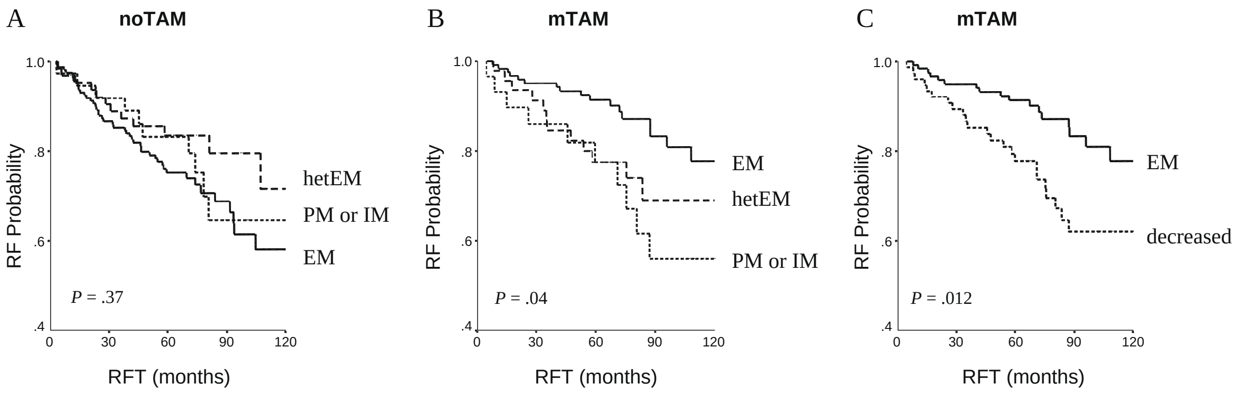

Fig. 2 – Kaplan–Meier estimates of relapse-free time (RFT) for CYP2D6 metaboliser phenotypes predicted from genotypes. (A) Patients not treated with tamoxifen (noTAM); (B) patients treated with adjuvant tamoxifen monotherapy (mTAM); (C) carriers of one or two impaired CYP2D6 alleles predictive for decreased enzyme activity were combined; EM, IM, PM, extensive, intermediate, poor metaboliser, hetEM, heterozygous extensive.[59]

genotypes ($n = 153$). In a small cohort of 21 patients with metastatic breast cancer and treated with tamoxifen, all six patients with progressive or stable disease lasting less than 24 weeks carried the *10/*10 genotype ($P = 0.0186$). The median time to progression for *CYP2D6**10/*10 patients was significantly shorter than that for all other genotypes (5.0 versus 21.8 months, $P = 0.0032$). The Chinese study by Xu et al.[61] investigated 152 patients with 20 mg/daily adjuvant tamoxifen for 5 years and a cohort of 141 patients not treated with tamoxifen. Overall genotype frequencies were 24% for *10 wt/wt (C/C), 28% for wt/*10 (C/T), and 48% for *10/*10 (T/T). At a median follow-up time of 63 months, carriers of the CYP2D6 *10/*10 genotype had a significantly worse disease-free survival (89% versus 96%, $P = 0.005$), an association that was not observed in the patient cohort not treated with tamoxifen. Moreover, among 37 patients taking tamoxifen for at least 4 weeks, 4-hydroxytamoxifen levels were significantly lower in CYP2D6*10 homozygous genotype carriers than in patients with homozygous CYP2D6 wt/wt genotype ($P = 0.04$). The Japanese study by Kiyotani et al.[62] investigated 67 patients treated with 20 mg/daily tamoxifen for 5 years with a median follow-up of 10 years. Frequencies were 29.9% for CYP2D6 *1/*1 (wt/wt), 34.3% for *1/*10 and 22.4% for *10/*10. Patients with a CYP2D6 *10/*10 genotype showed a significantly higher incidence of recurrence than those with a CYP2D6 *1/*1 genotype ($P = 0.0057$) or a combined patient group carrying at least one *1 allele ($P = 0.0031$ for trend). Although some of the sample sizes in the Asian studies were low, their findings of an implication of CYP2D6 genotypes predictive for tamoxifen outcome are in line with the studies from Europe[59] and the US.[57,58]

No favourable association of CYP2D6 genetics and tamoxifen outcome was reported in studies from the US, by Nowell et al. (162 patients with tamoxifen, 175 patients without tamoxifen), and Sweden, by Wegmann et al. (112 patients with 40 mg/daily tamoxifen, and mean follow-up of 10.7 years), respectively.[63,64] While Nowell et al. reported no association between CYP2D6 *4 and tamoxifen response or breast cancer prognosis,[63] Wegman et al. reported a decrease in the number of recurrences in tamoxifen treated patients who carried the CYP2D6 *4 variant (odds ratio (OR) = 0.28; 95% CI, 0.11–0.74; $P = 0.0089$).[64] Wegman et al. in addition investigated a cohort of 677 tamoxifen-treated postmenopausal patients, 238 of whom were randomised to 2 versus 5 years of treatment. Patients homozygous for CYP2D6 *4 had a significantly better disease-free survival compared to patients homozygous or heterozygous for the *1 allele ($P = 0.05$ and $P = 0.04$, respectively); however, this effect was not significant in multivariate Cox analysis ($P = 0.055$).[65]

So far, most available evidence in favour of a relationship between CYP2D6 variation and tamoxifen treatment outcome is derived from patients with mainly adjuvant tamoxifen treatment. However, preliminary evidence suggests that this relationship may also play a role in breast cancer chemoprevention as reported from the Italian tamoxifen trial including 5408 healthy hysterectomised women aged 35–70 years who were randomly assigned to receive 20 mg daily tamoxifen or placebo. In a nested case-control study including 46 women who developed breast cancer and 136 controls, the frequency of CYP2D6 *4/*4 genotype was significantly higher in women who developed breast cancer than in those who did not: all women (tamoxifen and placebo): 9% versus 1% $P = 0.015$); tamoxifen treated women: 15% versus 2% ($P = 0.04$).[66] Unexpectedly, hot flashes were reported for all three CYP2D6 *4/*4 allele carriers who developed breast cancer during tamoxifen treatment.

Finally, a small study of hereditary breast cancer patients being tumour suppressor mutation carriers of BRCA1 (47 patients) or BRCA2 (68 patients) and treated with tamoxifen suggested a relationship between CYP2D6 PM status and worse survival.[67] This relationship was observed for BRCA2 but not for BRCA1 carriers. Due to small numbers, as well as ER positive and ER negative patients being included in the analysis, further investigation will be needed to distinguish a pharmacogenetic effect from a poor prognosis effect.

2.3. *Effects of metabolite levels and drug interaction on tamoxifen efficacy and outcome*

It is clear that patients must complete a 5-year course of tamoxifen because 5 years of tamoxifen is superior to 1 or 2 years of adjuvant treatment. This principle is elegantly demonstrated in the overview analysis of clinical trials for premenopausal patients with ER positive breast cancer (Fig. 3).[15] Although, in general, rates of tamoxifen adherence are higher than those observed for other medications, discontinuation of adjuvant tamoxifen in older women with ER positive breast cancer has been evaluated. Randomised clinical trials of adjuvant therapy reported 5-year discontinuance rates of 23% and 40%[4,68], and the primary prevention trial reported a 5-year dis-

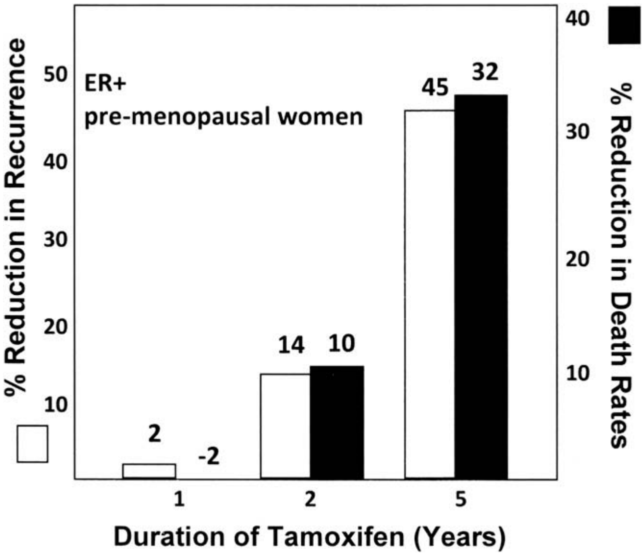

Fig. 3 – The influence of the durations of adjuvant tamoxifen therapy administered to premenopausal patients with oestrogen receptor (ER) positive (+) breast cancer.[15] The enhancement of a reduction of recurrences and a reduction of death rates between women taking only 1 year of adjuvant tamoxifen compared to 5 years serves to illustrate the benefits of the drug, the need for compliance, and the need to ensure that patients are neither poor metabolisers by virtue of aberrations of CYP2D6 or phenocopying by taking SSRIs to reduce menopausal symptoms.

continuance rate of 24%.[69] In clinical practice, discontinuation rates range from 15% to 50%.[70–74] Health-care data-based analyses revealed that as many as half of the patients stop their medication in the course of the 5-year adjuvant treatment with tamoxifen and as many as 15% and 22% of patients stop taking tamoxifen during the first year.[75–77]

The main obstacle to compliance is unacceptable side effects such as severe hot flashes and related menopausal symptoms.[70] However, there is accumulating evidence that hot flashes are an indicator of tamoxifen efficacy and, therefore, the patient's lack of compliance imposes an obstacle to successful treatment. This has recently been suggested by data from the Women's Healthy Eating and Living trial (WHEL)[78] which enrolled primary breast cancer patients ($n = 3088$ between 18 and 70 years of age) between 2 to 48 month after initial diagnosis to either dietary intervention ($n = 1537$) or observation ($n = 1551$) alone. At study entry, among the 864 women taking tamoxifen 78% reported hot flashes, and among those 69% also reported night sweats; only 4% reported night sweats without hot flashes, and 18% did not report either hot flashes or night sweats. Patients reporting hot flashes at baseline were less likely to develop recurrent breast cancer than those who did not report hot flashes (12.9% versus 21%, $P = 0.01$; 127 women had a confirmed breast cancer recurrence after 7.3 years follow-up). Moreover, hot flashes were more predictive of outcome than age, grade, hormone receptor status, or stage II cancer.[78] Goetz et al. showed that the incidence of hot flashes during adjuvant tamoxifen improved therapeutic outcomes and correlated with the CYP2D6 genotype.[57] In their study none of the patients with CYP2D6 *4/*4 genotype (0/13) reported moderate or severe hot flashes compared to 20% (36/177) in the groups of *4/wt and wt/wt patients ($P = 0.064$). Accordingly, hot flashes can be attributed to higher tamoxifen metabolite levels in patients with functional CYP2D6 and drug efficacy. These data which link the occurrence of hot flashes with CYP2D6 genotype and adjuvant tamoxifen outcome, clearly extend previous prospective cohort studies of adjuvant tamoxifen treatment that have already demonstrated that there is a wide inter-individual variability in the formation of tamoxifen metabolites and that steady-state endoxifen plasma concentrations during tamoxifen treatment are substantially reduced in women carrying CYP2D6 genetic variants.[23,29,56] Similar relationships have been reported in studies from Asia[60,61] and Europe.[79] Moreover, at the level of chemoprevention, higher levels of N-desmethyltamoxifen (i.e. endoxifen precursor, Fig. 1) have been reported for carriers of CYP2D6 variants after 1 year of tamoxifen, suggesting that the conversion into the clinically active endoxifen may be impaired.[80] In the light of these genotype–metabolite relationships it is of utmost importance that patients experiencing hot flashes sustain adjuvant tamoxifen despite the discomfort of adverse reactions.

To aid compliance, patients are routinely prescribed selective serotonin re-uptake inhibitors (SSRIs, Fig. 4) that reduce menopausal symptoms.[81–83] This, however, imposes a new challenge because it is known, that some SSRIs have a high affinity for the CYP2D6 enzyme[84,85] and, therefore, SSRIs can inhibit CYP2D6 activity and interfere with tamoxifen efficacy by blocking the conversion of tamoxifen to endoxifen. The relative inhibitory concentrations of SSRIs for the CYP2D6 enzyme product are noted in the legend of Fig. 4. While the effect of SSRIs on the plasma levels of endoxifen had been previously reported by Stearns et al.,[23] this endoxifen lowering effect has been subsequently linked to the patients' CYP2D6 genotype by Jin et al.[29] Plasma concentrations after 4 months of tamoxifen therapy were significantly lower in patients with a CYP2D6 homozygous variant (20 nM; 95% CI: 11.1–28.9 nM) or heterozygous genotype (43.1 nM, 95% CI: 33.3–52.9 nM) than those with homozygous wild type (78.0 nM; 95% CI: 65.1–90.1 nM) (both $P = 0.003$). In this study, 24 of the 78 patients took CYP2D6 inhibitors including paroxetine, fluoxetine, sertraline, citalopram, amiodarone and metoclopramide. Among patients who carried a homozygous wild type genotype, the mean plasma endoxifen concentration for patients using CYP2D6 inhibitors was 58% lower than that of patients not using SSRI co-medication (38.6 nM versus 91.4 nM, $P = 0.0025$), and in patients who were heterozygous

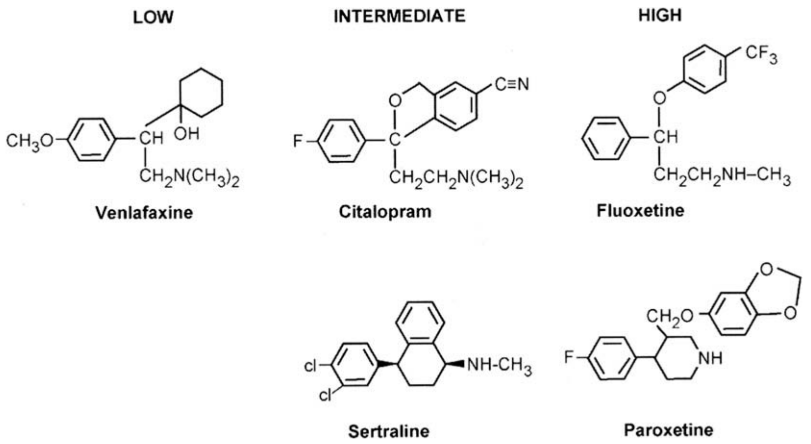

Fig. 4 – The selective serotonin re-uptake inhibitors (SSRIs) used to ameliorate hot flashes and menopausal symptoms during tamoxifen therapy. The SSRIs are CYP2D6 substrates and compete with N-desmethyltamoxifen for the CYP2D6 enzyme. Thy can be classified in high, intermediate, and low binding substrates for the CYP2D6 enzyme. The inhibitor constants for venlafaxine (low), citalopram (intermediate), and sertraline (intermediate), fluoxetine and paroxetine (high) are 33, 7, 1.5, 0.17 and 0.05, respectively.

for a non-functional CYP2D6 allele (wt/vt) this difference was 38% (31.0 nM versus 51.7, $P = 0.08$). Moreover, women taking the weak CYP2D6 inhibitor venlafaxine (a serotonine noradrenaline re-uptake inhibitor (SNRI)) had slightly reduced plasma endoxifen concentrations compared to women taking the potent CYP2D6 inhibitor paroxetine.[29] These findings suggest that both pharmacogenomic effects and pharmacological interactions of drugs at CYP2D6 have an influence on the metabolism of tamoxifen and, therefore, ultimately affect drug efficacy.

The extended investigations of Borges et al.[56] scrutinised the quantitative relationship between CYP2D6 variants, i.e. PM, IM and UM genotype, on endoxifen plasma concentrations in 158 patients at 4 months of 20 mg daily tamoxifen. They found that CYP2D6 genoptypes are highly associated with endoxifen plasma concentrations and account for their variability. While there were no significant differences in mean plasma concentrations of tamoxifen, N-desmethyltamoxifen and 4-hydroxytamoxifen between users and non-users of concomitant CYP2D6 inhibitors, the mean endoxifen plasma concentration was significantly lower in patients taking CYP2D6 inhibitors compared to that in patients who did not (39.6 ± 28.4 nmol/L versus 71.5 ± 41.2 nmol/L; $P < 0.01$).[56] When the authors divided the CYP2D6 inhibitors into potent (paroxetine, fluoxetine, $n = 19$) and weak (SSRI: sertraline and citalopram [$n = 14$] as well as celecoxib, diphenydramine and chlorpheniramine [$n = 13$]), they found a more pronounced decrease in mean endoxifen plasma concentrations with potent inhibitors than with weak inhibitors. Concomitant use of venlafaxine, which is considered the least potent inhibitor, did not show any significant effect. Taking into account CYP2D6 genotypes, the authors observed that the mean plasma endoxifen concentration was significantly lower in CYP2D6 EM patients who were taking potent CYP2D6 inhibitors compared to that in patients who were not (23.5 ± 9.5 nmol/L versus 84.1 ± 39.4 nmol/L, $P < 0.001$).[56] Thus, CYP2D6 genotype and concomitant potent CYP2D6 inhibitors are highly associated with plasma endoxifen concentrations and may substantially impact outcome during tamoxifen treatment by phenocopying effects i.e. converting an EM into a PM phenotype.

The phenocopying effect of SSRI with respect to their interplay with CYP2D6 genotype and effect on clinical outcome was explored by Goetz et al. in their recent follow-up of the NCCTG trial.[58] They investigated the role of CYP2D6 inhibitors in 256 patients that had been randomised to the tamoxifen alone arm. Patients with CYP2D6 wt/wt genotype who did not take CYP2D6 inhibitors were classified as EM ($n = 115$), whereas patients with either one or two *4 alleles or those taking a CYP2D6 inhibitor were classified as IM or PM ($n = 65$), depending on the strength of the inhibitor. Following these assignments, patients with decreased metabolism had shorter time to breast recurrence ($P = 0.015$), relapse-free ($P = 0.007$), disease-free ($P = 0.009$), and overall survival ($P = 0.082$) compared to those with extensive CYP2D6 metabolism.[58] The authors concluded that CYP2D6 metabolism, as measured by genetic variation and enzyme inhibition, is an independent predictor of breast cancer outcome in postmenopausal primary breast cancer patients receiving adjuvant tamoxifen. Accordingly, outcome during tamoxifen treatment may be influenced by its pharmacogenetics as well as co-prescription of drugs interfering with the CYP2D6 mediated tamoxifen metabolism.

3. Conclusion

In summary, we can conclude that endoxifen is formed by the CYP2D6 enzyme[21–23,28,35] and it is therefore anticipated that aberrant genotypes and other medicines that are metabolised by the same enzyme impair the actions of tamoxifen in patients.[29] We addressed the veracity of the hypothesis from the current literature to explore the possibility of targeting tamoxifen to improve women's health. There is now strong evidence that hot flashes are indicators of tamoxifen efficacy and that tamoxifen efficacy and outcome depend on the drug's metabolism which is subject to CYP2D6 genotype and pharmaco-interations. Data from numerous international studies[29,56–62] yielded consistent results in linking active tamoxifen metabolite plasma concentrations with genetically determined CYP2D6 metaboliser status, interference with strong CYP2D6 inhibitors, as well as clinical outcome. Few conflicting data[63–65] may be explained by variations in patient inclusion criteria into respective studies (e.g. variations in tamoxifen doses, length of treatment, additional chemotherapy regimens, lack of consistent ER testing). Importantly, most authors agree that genetic CYP2D6 variants, as well as CYP2D6 inhibition by prescribed co-medications such as antidepressants, may decrease tamoxifen metabolism, and thus negatively impact tamoxifen efficacy and treatment outcome.

There are a number of potential clinical consequences from these emerging data. First of all, strict compliance with tamoxifen treatment is critical for efficacy and outcome and, therefore, deviations from the prescribed course of adjuvant tamoxifen must be avoided even when side effects occur. Second, potent SSRIs such as paroxetine or fluoxetine should not be used for the relief of hot flashes in breast cancer patients receiving tamoxifen. Even though SSRIs are one of the few evidence-based therapy options for menopausal vasomotor symptoms,[86] available data indicate that they may compromise tamoxifen efficacy due to their interference with CYP2D6 dependent tamoxifen metabolism. Yet, this interference depends on the strength of the CYP2D6 inhibitor.[84,85] If treatment of hot flashes is indicated, a SSRI such as citalopram or escitalopram or a SNRI such as venlafaxine should be used because these substances showed no significant inhibition of CYP2D6.[29] Third, the CYP2D6 genotype/phenotype-treatment outcome relationship points to the possible benefit of upfront CYP2D6 genotyping prior to the initiation of endocrine treatment. A comprehensive robust, standardised, and quality controlled CYP2D6 genotyping test will need to analyse all relevant genetic variants that may affect tamoxifen metabolism which should include common PM alleles (*3, *4 and *5) and IM alleles depending on the individual's ethnic origin.[57–62] Of note, *41 is the most frequent IM allele in Europeans, *17 is the principal IM allele in Africans, and *10 dominates in Asians (in addition *9 should also be considered).[41] Other areas of interest with respect to clinical application are the measurement of endoxifen plasma levels as a surrogate of CYP2D6 phenotype and a possible dose increase of tamoxifen to overcome impaired CYP2D6 metabolism; however, the latter option requires further investigation before definite conclusions can be made.

Given alternative treatment options, i.e. tamoxifen versus aromatase inhibitors (AI), and considering the available scientific and clinical evidence, an individualised approach for endocrine treatment of postmenopausal breast cancer patients is desirable. One might speculate that tamoxifen alone may be adequate for CYP2D6 EM/EM (wt/wt) carriers whereas postmenopausal patients with variant CYP2D6 alleles may fare better with upfront AI therapy. However, currently, a formal recommendation on the integration of CYP2D6 genotypes in treatment decisions must await their validation in statistically powered and/or prospective clinical trials. While these may be under way it will be interesting to see whether the small difference in the outcome benefit of AI as compared to tamoxifen recently reported from landmark trials BIG 1–98[87] and ATAC[17,88] can be attributed to the lack of CYP2D6 genotype stratification. This possibility should be considered particularly in the light of insights from a biomathematical modelling exercise of the estimated benefit of adjuvant tamoxifen according to CYP2D6 gene status. Using the BIG 1–98 information on recurrence probabilities and assuming that AI metabolism was CYP2D6 independent, it has been suggested that the benefit of 5 years of adjuvant tamoxifen may even exceed that of upfront AI treatment in postmenopausal CYP2D6 wt/wt patients.[89] In the meantime, the International Tamoxifen Pharmacogenetics Consortium (http://www.pharmgkb.org/views/project.jsp?pId=63) is making an effort towards pooled analysis of available data to further strengthen our understanding of the relationship between CYP2D6 metabolism status and tamoxifen efficacy.

Finally, the personalised approach in targeting tamoxifen seems feasible and should await timely translation into clinical practise. Indeed, the CYP2D6 genotype might be one of the first predictors of therapeutic response in cancer care. Because this approach is genome-based by utilising CYP2D6 genotyping for the prediction of a patient's metaboliser phenotype, ethical issues need to be sufficiently addressed. In the light of acceptable alternatives, an informed choice about adjuvant endocrine treatment and, most importantly, avoidance of a therapy that might potentially lack efficacy must be prime interests. It will therefore be important to make patients and their care takers aware of these issues and also to initiate discussions with regulatory authorities.

Conflict of interest statement

None declared.

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Original Article

New hypotheses and opportunities in endocrine therapy: amplification of oestrogen-induced apoptosis

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SUMMARY

Aims: To outline the progress being made in the understanding of acquired resistance to long term therapy with the selective oestrogen receptor modulators (SERMs, tamoxifen and raloxifene) and aromatase inhibitors. The question to be addressed is how we can amplify the new biology of oestrogen-induced apoptosis to create more complete responses in exhaustively antihormone treated metastatic breast cancer.
Methods and Results: Three questions are posed and addressed. (1) Do we know how oestrogen works? (2) Can we improve adjuvant antihormonal therapy? (3) Can we enhance oestrogen-induced apoptosis?
The new player in oestrogen action is GPR30 and there are new drugs specific for this target to trigger apoptosis. Similarly, anti-angiogenic drugs can be integrated into adjuvant antihormone therapy or to enhance oestrogen-induced apoptosis in Phase II antihormone resistant breast cancer. The goal is to reduce the development of acquired antihormone resistance or undermine the ~~ability~~ resistance of breast cancer cells to undergo apoptosis with oestrogen respectively. Finally, drugs to reduce the synthesis of glutathione, a subcellular molecule compound associated with drug resistance, can enhance oestradiol-induced apoptosis.
Conclusions: We propose an integrated approach for the rapid testing of agents to blunt survival pathways and amplify oestrogen-induced apoptosis and tumour regression in Phase II resistant metastatic breast cancer. This Pharma platform will provide rapid clinical results to predict efficacy in large scale clinical trials.

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Introduction

Tamoxifen (ICI46,474) was not hailed as an impressive breakthrough in the early 1970's when it was marketed as an orphan drug that produced modest responses in the treatment of metastatic breast cancer in post menopausal women[1]. Only one in three tumours responded to treatment for about a year. Nevertheless, side effects with tamoxifen were less than other available endocrine therapies (diethylstilboestrol (DES) or androgens)[2–4].

Despite initial disinterest in endocrine therapy, significant progress was subsequently made in the treatment and chemoprevention of breast cancer through the clinical application of laboratory principles for the antihormonal therapy of breast cancer[5]. Today antihormonal therapies (tamoxifen and aromatase inhibitors) target the oestrogen receptor (ER) present in the majority of breast cancers and long term adjuvant therapy with tamoxifen increases patient survival[6,7]. Aromatase inhibitors that are used exclusively in postmenopausal patients improve disease-free survival when compared to tamoxifen, and reduced the risk of endometrial cancer and blood clots noted with tamoxifen[8]. Additionally, the application of SERMs for the chemoprevention of breast cancer either directly with tamoxifen and raloxifene[9,10] or indirectly with raloxifene for the prevention of osteoporosis[11–13] will surely reduce the incidence of breast cancer in select populations over the next decade.

The critical strategy that led to the success of endocrine therapy for the treatment and prevention of breast cancer was the implementation of the laboratory principle of extended durations of treatment[14,15]. However, the consequence of long term treatment is the development of antihormonal drug resistance. Numerous laboratory models of antihormonal drug resistance have been developed over the past 20 years and several valuable principles have emerged. Drug resistance with SERMs evolves through at least two distinct phases: Phase I and Phase II[16] (Fig. 1). Phase I resistance to tamoxifen treatment is characterized by either tamoxifen or oestradiol-stimulated growth. Both ligands can exploit the ER signal transduction pathway to aid tumour cell survival. This phase of drug resistance has a clinical equivalent in metastatic breast cancer. When treatment fails during tamoxifen therapy, the tumour has a

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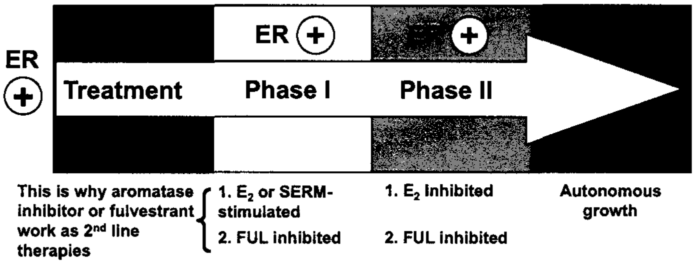
ER
⊕
Treatment
ER ⊕
Phase I
ER ⊕
Phase II
This is why aromatase inhibitor or fulvestrant work as 2nd line therapies
1. E2 or SERM-stimulated
2. FUL inhibited
1. E2 Inhibited
2. FUL inhibited
Autonomous growth

Fig. 1. The evolution of drug resistance to SERMs. Acquired resistance occurs during long-term treatment with a SERM and is evidenced by SERM-stimulated breast tumour growth. Tumours also continue to exploit oestrogen for growth when the SERM is stopped, so a dual signal transduction process develops. The aromatase inhibitors prevent tumour growth in SERM-resistant disease and fulvestrant that destroys the ER is also effective. This phase of drug resistance is referred to as Phase I resistance. Continued exposure to a SERM results in continued SERM-stimulated growth (Phase II), but eventually autonomous growth occurs that is unresponsive to fulvestrant or aromatase inhibitors. The event that distinguishes Phase I from Phase II acquired resistance is a remarkable switching mechanism that now causes apoptosis, rather than growth, with physiologic levels of oestrogen. These distinct phases of laboratory drug resistance[17,18] have their clinical parallels and this new knowledge is being integrated into the treatment plan.

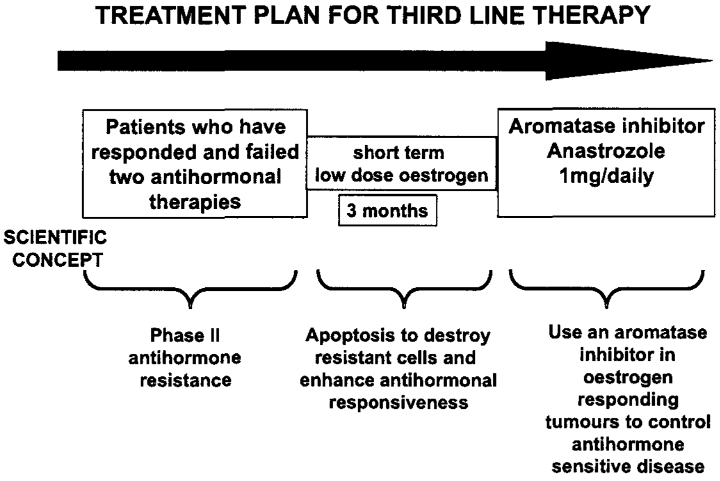
TREATMENT PLAN FOR THIRD LINE THERAPY
Patients who have responded and failed two antihormonal therapies
short term low dose oestrogen
3 months
Aromatase inhibitor Anastrozole 1mg/daily
SCIENTIFIC CONCEPT
Phase II antihormone resistance
Apoptosis to destroy resistant cells and enhance antihormonal responsiveness
Use an aromatase inhibitor in oestrogen responding tumours to control antihormone sensitive disease

Fig. 2. Clinical protocol to investigate the efficacy of oestradiol induced apoptosis in long-term endocrine refractory breast cancer. An anticipated treatment plan for third-line endocrine therapy. Patients must have responded and experience treatment failure with two successive antihormone therapies to be eligible for a course of low-dose oestradiol therapy for 3 months. The anticipated response rate is 30% and responding patients will be treated with anastrozole until relapse. Validation of the treatment plan will establish a platform to enhance response rates with apoptotic oestrogen by integrating known inhibitors of tumour survival pathways into the 3-month debulking "oestrogen purge". The overall goal is to increase response rates and maintain patients for longer on antihormone strategies before chemotherapy is required.

withdrawal response or regression upon withdrawal of tamoxifen treatment[19]. Second line therapy following tamoxifen treatment failure is with either an aromatase inhibitor or fulvestrant[20].

The description of Phase II resistance to tamoxifen was first presented at the St. Gallen Breast Cancer Conference in 1992[21]. Re-transplantation of tamoxifen-resistant MCF-7 breast tumours into tamoxifen treated athymic mice for 5 or more years causes the signaling networks through the ER, that normally act as a survival network, to become reconfigured to be activated by physiological oestradiol that causes rapid apoptosis and triggers tumour regression[17]. The fact that these laboratory data pertaining to the evolution of drug resistance to tamoxifen also applies to antihormonal resistance to raloxifene[22], and oestrogen withdrawal[23–25] creates a valid general principle in breast cancer that can now be exploited in the clinic to enhance patient survivorship[18,26]. Indeed, it has been suggested that these current data[23,27] can explain the effectiveness of high dose oestrogen therapy to treat metastatic breast cancer in post menopausal women before tamoxifen was available[28]. High dose DES produces a 35% response rate in unselected patients[29] and interestingly enough Lonning and colleagues[30] reported a 30% response rate for high dose DES in a population of women who have received exhaustive antihormonal therapy to treat metastatic breast cancer. Remarkably, one woman has had a complete remission for more than 8 years after starting DES treatment (Per Lonning, personal communication).

We now choose to amplify the clinical potential of short term low oestradiol therapy to treat breast cancer in those patients whose ER positive tumour has responded and failed at least two consecutive antihormonal therapies. Based on the emerging clinical experience and on an expanding laboratory data base we anticipate a 30% clinical benefit[30]. We address the question of why tumour cell survival signaling prevents 70% of Phase II tumours from responding to low dose oestradiol and we will advance short and long term solutions to apply pharmacological interventions to sensitize refractory breast cancers to oestradiol's apoptotic actions.

A clinical model to evaluate oestrogen induced apoptosis

We have previously proposed a clinical test bed to define the molecular biology and breast tumour responsiveness to both high doses (30 mg daily) and low dose (6 mg daily) oestradiol. That strategy is based on the translation of our laboratory description of the evaluation of anti-hormone resistance through phase I to phase II resistance where oestradiol switches from being a survival signal to an apoptotic trigger[16,27,31]. The schema for the trial is illustrated in Fig. 2. Breast cancer patients who are eligible for recruitment to the trial must have responded and failed two consecutive anti-hormonal therapies e.g. fail tamoxifen adjuvant therapy during year 3–5 and subsequently respond and fail an aromatase inhibitor during the treatment of metastatic breast cancer. In our first protocol of high dose oestradiol therapy (30 mg daily), we are treating for 12 weeks and then responding patients will be treated with an aromatase inhibitor (anastrazole 1 mg daily) until progression[32]. Several other investigators have initiated similar clinical trials but with less rigorous entry criteria concerning failure of repeated endocrine therapies. Nevertheless, we contend that the moment is right to address the issue of the regulation of apoptosis and advance the idea that other agents may be synergistic with oestradiol to trigger apoptosis in the predicted 70% of patients that do not respond to short term oestradiol therapy.

Opportunities in the endocrine regulation of breast cancer

Naturally, it is not possible to consider all of the opportunities that could be exploited for patient benefit but we pose three questions that will be answered with an example of current research from our laboratory. The questions will be addressed as an integrated translational research scheme summarized in Fig. 3.

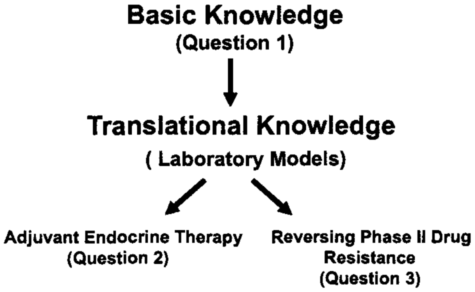

Fig. 3. The interactive translational research model employed to address new hypotheses and opportunities to amplify oestrogen-induced apoptosis for the treatment of Phase II endocrine resistant breast cancer. The questions posed are described in the text.

Question 1: Do we know how oestrogen works in target tumours?

The ER with its modulation through co-activators and co-repressors[33] has been investigated extensively through the structure function relationships of ligands that create novel folding of the receptor complex[34]. However the array of SERMs is only able to add marginally to advancing cancer therapeutics. We are beginning to understand the cross talk between the ER pathway and growth factor receptor pathways but our basic knowledge is in its infancy. There is an increasing menu of medicines to block growth factor pathways, but the challenge is to place the right targeted agent in the endocrine treatment paradigms. We will return to this challenge in our summary.

Our confidence in the position that "we understand how oestradiol works" has been challenged twice in recent times firstly with the discovery of a second ER referred to as ER-β (ER-α is the classical ER), and secondly with the discovery of the G protein-coupled receptor GPR30, an oestrogen-, SERM- and fulvestrant-binding protein. The role of ER-β in breast cancer is controversial but there is evidence that overexpression of ER-β can inhibit proliferation[35] and cause apoptosis[36]. However, ER-β specific ligands have yet to find a role in cancer therapeutics. The G protein-coupled receptor GPR30 is the latest putative receptor that can modulate oestrogen action specifically[37]. The molecule, a seven-pass transmembrane receptor located in the endoplasmic reticulum, mediates rapid non-genomic actions of oestradiol to initiate mobilization of intracellular Ca^{++} stores.

Based on high through put screening assays a new class of molecules specific for GPR30 has been identified[38], and one compound G1 (Fig. 4) is available for laboratory investigations. We have addressed the question of whether the GPR30 agonist G1 is a stimulator or blocker of oestradiol-stimulated growth in the wild-type ER-positive MCF-7 breast cancer cell line and whether G1 can provoke apoptosis in our oestrogen deprivation-resistant cell lines MCF-7:5C[24] and MCF-7:2A[39]. Data shown in Fig. 5 illustrates the fascinating pharmacology of the new drug group. G1 is anti-oestrogenic in the wild type MCF-7 cell line, and enhances apoptosis in both the MCF-7:5C and MCF-7:2A cell lines. Most importantly, G1 induces apoptosis in the MCF-7:2A cells more rapidly than oestradiol. This is important as it provides evidence that in endocrine resistant breast cancer cells, which are initially refractory to the immediate apoptotic actions of oestradiol, there is the potential to circumvent survival and initiate apoptosis quickly via a new pathway. The mechanism of action of G1 in all breast cancer cell lines is the rapid mobilization of high levels of Ca^{++} from intracellular stores. This increase of Ca^{++} is cytotoxic, thus, the new drug group has potential to enhance apoptosis in anti-hormone resistant cell lines and further development of these agents may find an application for short term treatment of patients whose tumours are Phase II anti-hormone resistant.

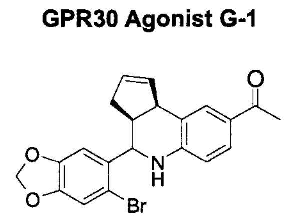

Fig. 4. G1, the first of a new class of agents that act as selective agonists of GPR30. A range of antagonists is also being developed.

Question 2: Can we improve adjuvant antihormonal therapy?

We have probably reached a zenith with what can be achieved with adjuvant antihormonal therapy. Nevertheless, significant increases in efficacy can be achieved by improving compliance for long term adjuvant therapy or selecting out those patients that have variant CYP2D6 that does not metabolize tamoxifen to the active metabolite endoxifen[40]. What is required is a new initiative that can significantly enhance the effectiveness of antihormonal therapy and reduce the development of acquired drug resistance and possibly block intrinsic resistance. It could be, that the 40% of ER positive breast cancers that do not respond initially to antihormones could be encouraged to do so by pharmacologic intervention.

Angiogenesis is critical for the growth of tumours and the establishment of metastatic lesions[41]. However, antiangiogenic drugs must be integrated into the cancer treatment plan as there are no advantages to monotherapy. As a result there is increasing interest in combining antiangiogenic drugs with cytotoxic chemotherapy with the goal of achieving better tumour responses[42]. There has however, been little interest in combining antiangiogenic agents with antihormonal therapy primarily because such long term treatments are required and the effective doses of antiangiogenic drugs have significant side effects that are often life threatening.

The development of acquired resistance to SERMs implies that angiogenic mechanisms must be activated in cancer cells to permit SERM stimulated growth. Indeed, recent research has demonstrated that an autocrine Vascular Endothelial Growth Factor (VEGF) VEGF receptor 2 (VEGFR2) and P38 signaling loop confers resistance to 4-hydroxytamoxifen in MCF-7 breast cancer cells[43]. Thus, the rationale of combining antihormonal therapy with antiangiogenic therapy has conceptual merit.

We have addressed the idea that low doses of an inhibitor of the VEGFR2 tyrosine kinase could be synergistic with tamoxifen to enhance the control of tumour cell growth in vivo. There is merit to using low doses of small molecule inhibitors of VEGFR2 in treatment regimens as side effects will be reduced and the drug may be sufficient to block the modest, but significant, angiogenic

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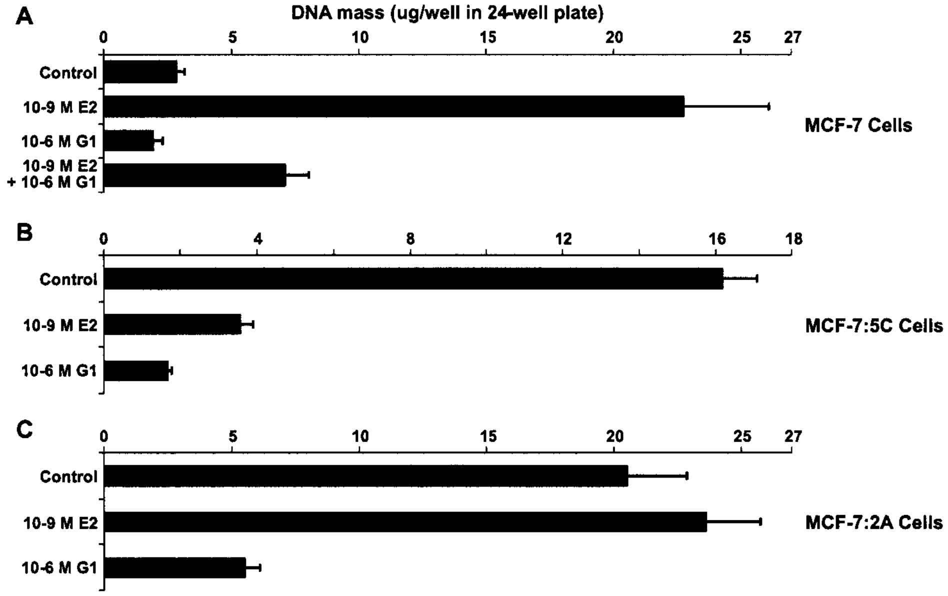

Fig. 5. The selective GPR30 agonist G1 inhibits growth of **(A)** wild-type MCF-7 cells and of oestrogen deprivation-resistant **(B)** MCF-7:5C and **(C)** MCF-7:2A cells. Cells were cultured under oestrogen-free conditions for 4 days, and then seeded into 24-well plates. Wild-type MCF-7 cells were seeded at 15,000 cells per well, MCF-7:5C cells at 25,000 cells per well, and MCF-7:2A cells at 30,000 cells per well. Beginning 24 hours after seeding (day 0) and every 2 days thereafter up to 6 days (days 2, 4, and 6), the cells were treated with 1 nM E_2, 1 μM G1, 1 nM E_2 + 1 μM G1, or Control (0.1% EtOH)-treated. The experiment was stopped on day 7. As a measure of proliferation, the amount of DNA per well was determined using a fluorescence-based DNA quantitation assay (CyQuant GR, Invitrogen, Carlsbad, CA). Data are shown as the mean of 8 replicate wells per group ± SD. **(A)** In wild-type MCF-7 cells, G1 significantly inhibited E_2-stimulated growth by 78% (E_2 vs. E_2+G1, $P<0.0001$), and inhibited growth relative to control-treated cells (control vs. G1, $P=0.0003$). **(B)** In estrogen deprivation-resistant MCF-7:5C cells, E_2 induced apoptosis as expected leading to a 78% reduction in growth (control vs. E_2, $P<0.0001$). G1 also significantly inhibited growth by 90% (control vs. G1, $P<0.0001$), and further, was more potent than E_2 (G1 vs. E_2 $P<0.0001$). **(C)** The oestrogen deprivation-resistant MCF-7:2A cells grew independently of E_2 within the 7 day course of the experiment, as expected, yet G1 significantly inhibited growth by 73% ($P<0.0001$).

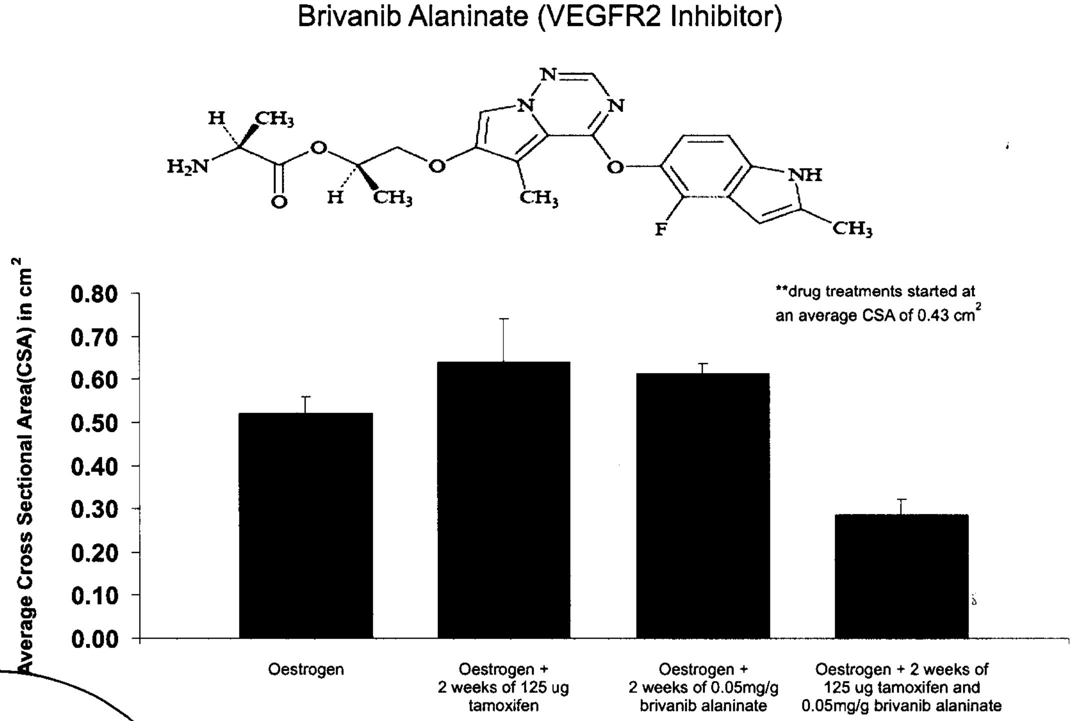

Fig. 6. Established MCF-7 E2 tumours and their response to various drug treatments. Tumours were implanted bilaterally into the mammary fat pads of athymic mice and 0.3 cm estradiol capsules were implanted subcutaneously into the dorsum of each mouse. Tumours were grown to 0.43 cm^2 and then drug treatments were initiated. Tumours that were treated with 125 ug of tamoxifen or 0.05 mg/g brivanib alaninate were unable to overcome oestradiol stimulated growth ($p=0.65$, $p=0.21$). Tumours continued to grow in the presence of oestrogen. When 125 ug of tamoxifen was combined with 0.05 mg/g brivanib alaninate, the effect was synergistic ($p=0.009$) and the tumours decreased in size. The tumours were 38% smaller than the oestrogen treated tumours, even though the observed difference was not significant ($p=0.16$). However, the decrease in average cross sectional area was significant when comparing the combination treatment to tamoxifen treated tumours ($p=0.01$) or those treated with brivanib alaninate ~~(p=007)~~. ($P = 0.007$)

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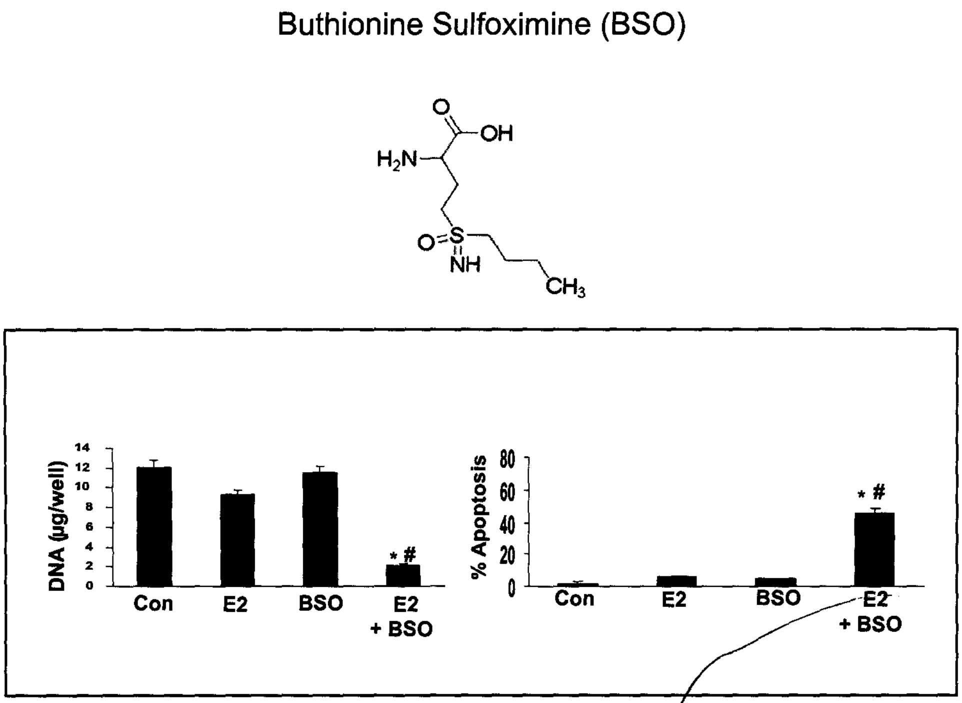

Fig. 7. The combination treatment of BSO plus oestradiol inhibits the growth of antihormone-resistant MCF-7:2A breast cancer cells. MCF-7:2A cells (30,000/well) were seeded in 24-well plates and after 24 hours were treated with <0.1% ethanol vehicle (control), 1 nM E2 (E2), 100 µM BSO (chemical structure shown above), or 100 µM BSO plus 1 nM E2 for 7 days. At the indicated time point, cells were harvested and total DNA (µg/well) was quantitated ~~as described in Materials and Methods. The~~ These data represent the mean of three independent experiments; *bars*, ±SE. $^{**}P < 0.001$ compared with control cells; $^{\#\#}P < 0.001$ compared with oestradiol-treated cells. Annexin V staining for apoptosis was performed in MCF-7:2A cells following BSO plus E2 treatment. Quantitation of apoptosis (percent of control) in the different treatment groups is shown on the *right*. bars, ±SEs. $^{*}P < 0.05$ compared with control cells; $^{\#}P < 0.01$ compared with oestradiol-treated cells.

action of tamoxifen. In preliminary studies, we show (Fig. 6) that a combination of tamoxifen and a VEGFR2 inhibitor brivanib alaninate is superior to tamoxifen alone at inhibiting oestradiol induced tumour growth in athymic animals. The low dose of brivanib alaninate used does not significantly affect oestradiol-stimulated tumour growth when used alone. We conclude that the angiogenic signal from oestradiol is too strong but that the inhibition of the cell cycle with tamoxifen and the antiangiogenic brivanib alaninate in combination is synergistic.

The issue to be addressed is how to test the concept before committing to large scale adjuvant trials. One approach would be to evaluate efficacy and safety in our proposed model of oestradiol induced apoptosis in Phase II resistant breast cancer (Fig. 2). The goal would be to evaluate short term antiangiogenesis treatment by limiting toxicity during the 12 week treatment period and to assess improvements in response rates to physiologic (6 mg dose) oestradiol treatment alone. This would also address the third question we pose.

Question 3: Can we enhance oestrogen-induced apoptosis?

An effective treatment strategy for breast cancer must have a clear goal with the aim of enhancing patient survivorship. The progress[44] being made by translating the laboratory studies[45] of low dose apoptotic oestradiol therapy into clinical practice must be amplified to bring further benefits to a select group of patients. Those patients with Phase II resistant metastatic breast cancer are a significant proportion of all those who respond initially to adjuvant endocrine therapy. The goal is to harness the apoptotic trigger and create an enhanced sensitivity to oestrogen so that a higher proportion of tumours have a complete response to treatment. An application of general pharmacologic principles can be seen as a first step in amplifying oestrogen-induced apoptosis. Inhibitors of angiogenesis would be a logical innovation to aid oestrogen-induced apoptosis. By denying the ability of resistant cells to grow by restricting angiogenesis, may result in cellular instability and to enhance sensitivity to apoptosis. However, it is the critical players in the inhibition of apoptosis that need to be targeted in a broad strategy of combination therapy. It is generally agreed that Bcl-2 plays a central role in preventing the intrinsic apoptosis trigger through the mitochondrial pathway of cytochrome C release. One mechanism by which Bcl-2 may function is as an anti-oxidant by up-regulation of glutathione leading to rapid detoxification of reactive oxygen species and inhibition of free-radical mediated mitochondrial damage.

Glutathione is a water soluble tripeptide composed of glutamine, cysteine, and glycine. Elevated levels prevent apoptotic cell death whereas depletion of glutathione facilitates apoptosis[46] L-Buthionine sulfoximine (BSO) (Fig. 7) is a specific inhibitor of glutanylcysteine synthase that blocks the rate limiting step of glutathione biosyntheses.

Recent laboratory studies demonstrate[39] that oestrogen deprived MCF7 cells that are initially refractory to oestradiol induced apoptosis are sensitized to the immediate apoptotic action of oestrogen by BSO at concentrations that can be achieved clinically[47] (Fig. 7). Since there is an extensive clinical experience with BSO it would not be unreasonable to integrate the antioxidant concept into the clinical test model[48].

An integrated clinical strategy to target survival pathways in Phase II breast cancer

Overall, we are making significant progress towards understanding oestrogen-induced apoptosis and there is evidence that a new drug group based on GPR30 agonists could be developed to provide additional specificity and induce apoptosis in breast cancer. This approach could overcome some resistance in tumour cells observed

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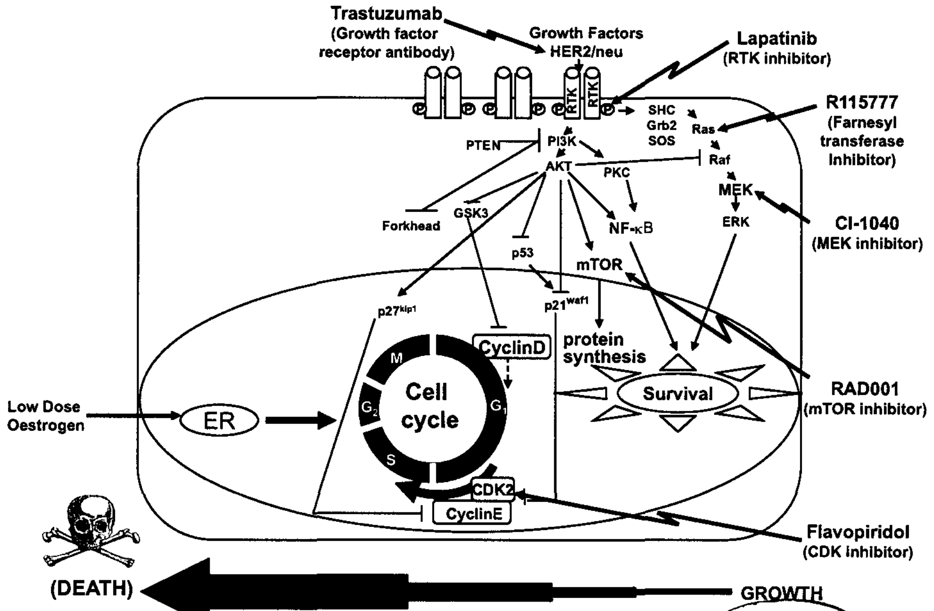

Fig. 8. Hypothetical apoptosis enhancement strategy to amplify the tumouricidal action of low dose oestradiol treatment. The strategy is to employ targeted agents from ~~the pharmacological~~ pharmaceutical industry to block several pathways and shift the cellular equilibrium to apoptosis in oestrogen refractory Phase II resistant cells. The diagram illustrates candidate drugs to create a cocktail in the proposed Pharma platform. Drugs would be tested singly with oestradiol against oestradiol alone or in increasing combinations of survival inhibitors.

with oestradiol alone. This, however, is a long term goal and builds on an evolving understanding of the complexities of oestrogen action in cancer. Similarly the anti-angiogenic drugs that block the tyrosine kinase activity of VEGFR2 could undermine the survival of tumours that are refractory to the apoptotic oestrogen trigger. But in practical terms, the application of BSO with an apoptotic oestrogen trigger has immediate clinical applications in our clinical test model (Fig. 2). We have presented developing laboratory evidence to support each of these pharmacologic strategies to amplify the apoptotic oestrogen therapy in Phase II resistant breast cancer. However, the mechanism based clinical test model is more than a translational research tool.

Rapid clinical results can be developed through mechanism based targeting of several pathways that have the potential to amplify the apoptotic oestrogen trigger to create a significant increase in complete tumour responses. In Fig. 8 are examples of agent classes that could potentially be tested rapidly in the 12 week model against oestrogen alone. This will establish efficacy of a new targeted agent as a clinically useful drug.

The hypothetical, yet systematic, strategy to evaluate selectively the inhibition of survival signals has a foundation in laboratory science. The obvious strategy of blocking the growth factor receptor signal cascade using either trastuzumab or the tyrosine kinase inhibitor lapatinib as an immediate practical approach in the 12 week test model. Recent studies demonstrate that antihormone responsiveness can be restored by aromatase resistant cells using trastuzumab[49] and lapatinib[50] is showing promise in clinical trials of breast cancer with chemotherapy[51].

The mammalian target-of-rapamycin (mTOR) is emerging as an important target for therapeutic intervention in multiple cancer tissue types including breast cancer. mTOR integrates signals from multiple pathways to sense cellular nutrient and energy levels. mTOR is a serine/threonine kinase downstream of PI3K/Akt that, in the presence of mitogenic stimulation and sufficient nutrients, promotes protein translation by activating 40S ribosomal protein S6 kinases (S6K1-2) and inhibiting the eukaryotic initiation factor 4E binding proteins (4E-BP1-3)[52]. RAD001 (everolimus) is an orally available mTOR inhibitor that alone and synergistically in combination with the aromatase inhibitor letrozole[53] blocks proliferation and induced apoptosis in MCF-7 and T47D breast cancer cells stably expressing aromatase[53]. These and other data have lead to the evaluation of RAD001 in combination with letrozole in a recently completed Phase I clinical trial in patients with advanced breast cancer[54]. RAD001 is also currently under evaluation in 15 other breast cancer clinical trials (search of clinicaltrials.gov on 2/26/2009) either as a single agent or in combination with various chemotherapeutics, fulvestrant, aromatase inhibitors, and agents which target EGFR and HER2/ErbB2.

We evaluated RAD001 in an MCF-7 breast cancer xenograft tumour model (MCF-7/E2) grown in athymic mice (Fig. 9A) that represents the therapeutic stage of antihormone-based therapy, and in a SERM-resistant (MCF-7/RAL1) xenograft tumour model (Fig. 9B) that was selected *in vivo* by continuous treatment with the SERM raloxifene for greater than 3 years[31,55]. RAD001 inhibited MCF-7/E2 tumour growth in the presence of E_2 (E_2 + RAD001 vs E_2 alone). Additionally, RAD001 in the absence of E_2, a situation comparable to combination therapy of RAD001 plus an aromatase inhibitor in the clinic, further reduced MCF-7/E2 growth (RAD001 alone vs. E_2 + RAD001). These MCF-7/RAL1 tumours can be considered cross-resistant to oestrogen deprivation (or aromatase inhibitors). However, RAD001 was still effective at blocking growth despite resistance to oestrogen deprivation (RAD001 vs. vehicle). Fulvestrant can be used clinically as a second-line therapy after failure of a first-line antihormone therapy, as illustrated here by fulvestrant inhibiting growth in the MCF-7/RAL1 tumours (fulvestrant vs. vehicle or RAL). Yet the combination of RAD001 plus fulvestrant was superior at blocking growth than either agent alone (Fulvestrant + RAD001 vs. fulvestrant alone and vs. RAD001 alone). Taken together, RAD001 represents a promising therapeutic for use in antihormone-sensitive, and importantly, in antihormone-resistant breast cancer, especially in combination with fulvestrant.

It is clear that other inhibitors of signal transduction pathways (Fig. 8) may be useful to enhance estrogen-induced apoptosis such as the MEK inhibitor CI-1040[56], the farnesyl transferase inhibitor lonafarnib[57] and the cyclin-dependent kinase inhibitor flavopiridol[58]. Indeed, inhibitors of CDK may have merit as a short term blocking strategy to enhance apoptosis. The cyclin-dependent kinase inhibitory drugs such as flavopiridol that have

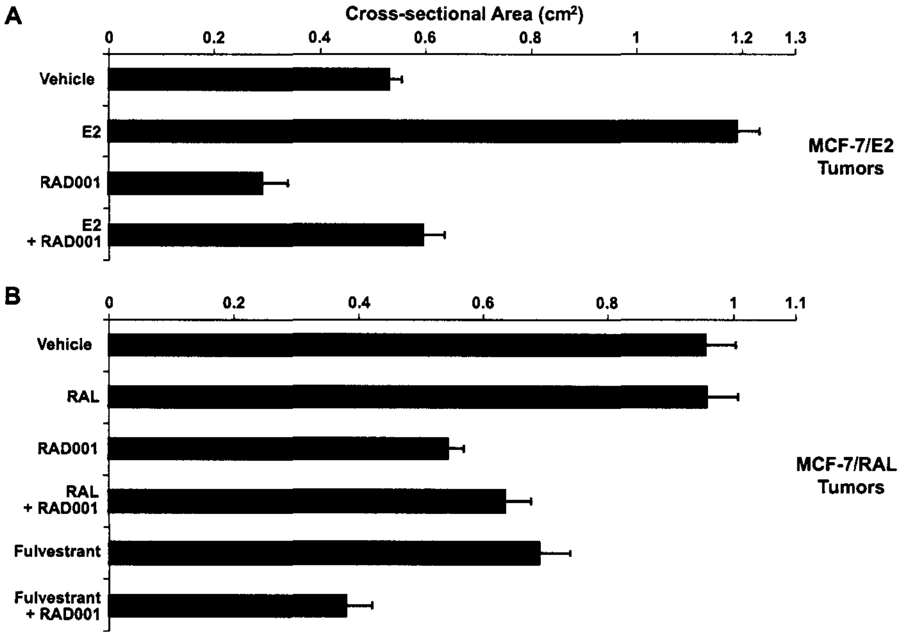

Fig. 9. Growth inhibition of naïve MCF-7/E_2 tumours and SERM-resistant MCF-7/RAL1 tumours in response to RAD001 (everolimus). **(A)** *RAD001 inhibition of MCF-7/E_2 tumour growth.* Twenty ovariectomized athymic nude mice were bilaterally transplanted with MCF-7/ E_2 tumour pieces 1 mm³ in size in the axillary mammary fat pads, and implanted with a 0.3 cm E_2 silastic capsule *sc*. Once the tumours grew to an average cross-sectional area of 0.39 cm², the animals were randomized into 4 treatment groups of 5 mice per group (10 tumours per group) corresponding to Vehicle (of the RAD001 formulation), E_2 (0.3 cm E_2 capsule *sc*), RAD001 [40 mg/kg/day (6.25 mg/day) RAD001 given 5 days/week], and E_2 + RAD001 (0.3 cm E_2 capsule *sc* plus 6.25 mg/day RAD001 given 5 days/week). The average cross-sectional area of RAD001-treated MCF-7/E_2 tumours was significantly smaller than Vehicle-treated tumours ($P = 0.0066$, T test). Similarly, the average cross-sectional area of E_2 + RAD001-treated tumours was significantly smaller than E_2 alone-treated tumours ($P < 0.0001$). **(B)** *RAD001 inhibition of MCF-7/RAL1 tumour growth.* Thirty ovariectomized athymic nude mice were bilaterally implanted in the axillary mammary fat pads with 1 mm³ MCF-7/RAL1 tumour pieces. Mice were treated with 1.5 mg/day RAL *po* until the MCF-7/RAL1 tumours grew to an average cross-sectional area of 0.26 cm², and then the animals were separated into 6 treatment groups of 5 mice each (10 tumours per group) corresponding to Vehicle (of the RAD001 formulation), 1.5 mg/day RAL *po*, RAD001 (6.25 mg/day RAD001 given 5 days/week), RAL + RAD001 (1.5 mg/day RAL *po* plus 6.25 mg/day RAD001 given 5 days/week), Fulvestrant (2 mg/day *sc* of the clinically used Faslodex preparation given 5 days/week), Fulvestrant + RAD001 (2 mg/day Faslodex *sc* plus 6.25 mg/day RAD001 given 5 days/week). The average cross-sectional areas of RAD001-treated and Fulvestrant-treated tumours were each significantly smaller than Vehicle-treated tumours ($P < 0.0001$ and $P = 0.0015$, respectively). Similarly, RAL + RAD001-treated tumours were significantly smaller than RAL-treated tumours ($P = 0.0002$). Additionally, Fulvestrant + RAD001-treated tumors were significantly smaller than RAD001 alone-treated tumors ($P = 0.0026$) or Fulvestrant alone-treated tumors ($P = 0.0004$). The data shown represent the average cross-sectional tumour area (cm²) per group ± SE. Tumour cross-sectional area was calculated using the equation $(l/2) \times (w/2) \times \pi$. The cross-sectional areas of MCF-7/E2 tumours were compared at day 41, and of MCF-7/RAL1 tumours at day 54.

been tested clinically and causes apoptosis through an intrinsic pathway dependent on BAX and BAK[58] would be of significant interest in combination with oestradiol to amplify apoptosis.

In summary a whole spectrum of new compounds can now be tested to enhance tumour response to oestrogen with the added advantage that this testing platform can document rapid tumour responses. Combinations could create an optimal cocktail for individual tumours to predict a complete response triggered by oestrogen.

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